# Identifying genes that underlie evolution of *Antirrhinum* species

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## Abbreations

| | |
|---|---|
| **AFLP** | Amplified fragment-length polymorphism |
| ***ant*** | *Aintegumenta* |
| ***AS1*** | *ASYMMERRIC LEAVES1* |
| ***axr1*** | *Auxin-resistant1* |
| ***BB*** | *Big Brother* |
| **bHLH** | Basic-helix-loop-helix |
| **bp** | Base pairs |
| **CAPS** | Cleft amplified fragment polymorphisms |
| ***CEN*** | *centroradialis* |
| ***cin*** | *cincinata* |
| **CK** | Cytokinin |
| ***CLV1*** | *CLAVATA 1* |
| ***CLV3*** | *CLAVATA 3* |
| ***DCL1*** | *Dicer-like1* |
| **DNA** | Deoxyribonucleic acid |
| **dNTPs** | Deoxynucleotide triphosphates mix |
| **EDTA** | Ethylenediaminetetraacetic acid |
| ***EGL3*** | *Enhancer of Glabra3* |
| **ER** | Erecta |
| ***FIL*** | *Filamentous flower* |
| ***FLO*** | *Floricula* |
| **GA** | Gibberellic acid |
| ***GL1*** | *Glabra1* |
| ***GL2*** | *Glabra2* |
| ***Hirz*** | *Hirzina* |
| **IEB** | Institute of Evolutionary Biology |
| **IMPS** | The Institute of Molecular Plant Sciences |
| ***Ina*** | *Invaginata* |
| ***KAN*** | *KANADI* |
| **kbp** | kilo base pairs |
| ***kn1*** | *Knotted1* |
| ***KNOX*** | *Knotted1-like homeobox* |
| ***LFY*** | *Leafy* |
| ***ls*** | *Lateral suppressor* |
| **miRNAs** | Micrornas |

| | |
|---|---|
| **NsPHAV** | Nicotiana sylvestris PHAVOLUTA |
| ***nt*** | *Nucleotide* |
| **NTH15** | Nicotiana tabacum Homeobox15 |
| **PCR** | Polymerase chain reaction |
| ***PHAN*** | *Phantastic* |
| **PHB** | *Phabulosai* |
| **PHV** | *Phavoluta* |
| **QTL** | Quantitative trait locus |
| **RAPD** | Random Amplification of Polymorphic DNA |
| **RFLP** | Restriction fragment length polymorphism |
| **RNA** | Ribonucleic acid |
| ***Ros1*** | *Rosea1* |
| ***Ros2*** | *Rosea2* |
| **SAM** | Shoot apical meristem |
| ***STM*** | *SHOOTMERISTEMLESS* |
| **TALE** | Three Amino acid Loop Extension |
| ***tb1*** | *The teosinte branched 1* |
| **TBE** | Tris/boric acid/EDTA buffer |
| **TE** | Tris/EDTA buffer |
| ***TFL1*** | *Terminal flower1* |
| ***TTG1*** | *Transparent testa glabra1* |
| ***UFO*** | *UNUSUAL FLORAL ORGANS* |
| ***UNI*** | *Unifoliata* |
| **UV** | Ultra violet light |
| ***Ve*** | *Venosa* |
| **WT** | Wild type |
| ***WUS*** | *WUSCHEL* |
| ***YAB*** | *YABBY* |
| ***YAB3*** | *Yabby3* |

## Abstract

In nature, many differences between species are quantitative. To study the genetic basis for shape and size differences between plant species, *Antirrhinum* was used as a model. *Antirrhinum* provides great morphological variation between species and species can be crossed to each other to make hybrid progenies. *A. majus*, which has larger organs, smooth leaves and erect stems, was crossed with *A. molle*, which has smaller organs, very hairy leaves and is very branched, to produce an F2 hybrid population which varied for these traits.

QTL mapping provides a powerful approach to look at correlation between genotype and phenotype. A genetic map of the F2 population was therefore constructed, containing 192 markers in eight linkage groups, and used for quantitative trait loci (QTL) analysis. More than 70 different morphological traits were analyzed for correlation to compare the variance within and between organs and individuals. Eighty significant QTLs were detected for 25 traits, averaging three loci per trait. For two traits, candidate genes for QTL were examined. Each locus explained 7.7% - 74.0% of the variance in the trait (average 18.5%). Loci together explained 29.1% - 98.9% of the total variance in each trait (average 57.2%). The loci were spread throughout the genome and most loci (61.6%) had co-dominant alleles (i.e. both are likely to be active to different degrees). Almost all traits in the F2 population had positive and negative effects for QTL, suggesting that there has not been a lot of directional selection during the speciation of *A. majus* and *A. molle*.

# Chapter 1: INTRODUCTION

## Morphological Variation in Natural Populations

The morphological variation within or between different species is the result of mutation, selection and drift. Much natural variation in morphology and development within species or between closely related species is continuous, due to the different contributions from numerous genes acting in a quantitative way (Kroymann and Mitchell-Olds, 2005). Some traits in natural populations, however, are determined by loci with major effects that segregate as Mendelian traits. The effects of these loci can be seen directly from phenotypes, such as flower colour or shell colours and patterns in snails (Nadeau, 2001; Glazier *et al.*, 2002).

For other traits, the phenotype can be complicated by non-additive interactions between genes (e.g. epistasis) and by interaction with the environment. These interactions further reduce the ability to relate phenotype to genotype (Glazier *et al.*, 2002). Although knowledge of the genetic basis of phenotypic variation among organisms is central to understanding evolutionary adaptations underlying natural and artificial selection, the identification and isolation of the genes underlying these variations has been difficult (Frary *et al.*, 2000).

### • The Genetics of Complex Traits

Quantitative genetics aims to understand the genetic basis for complex traits. Traditionally this involved estimating the number and effect of loci from, for example, parent-offspring correlations or response to directional selection (Falconer and MacKay, 1996). Although attempts had been made to identify the positions and effects of loci contributing to quantitative variation, though linkage to morphological markers (Thoday, 1961.), the discovery of high-throughput genotyping technologies allowed high-resolution mapping of complex traits relative to naturally occurring DNA sequence variation (Botstein *et al.*, 1980).

### • Finding the Molecular Basis of Quantitative Traits.

The genetic architecture of a quantitative trait is often assumed to consist of a large number of genes (Barton and Turell, 1989); each with

a small contribution to the phenotype. After the development of different type of high-density genetic maps of polymorphic markers, it became possible to dissect quantitative trait loci in many species (Tanksley, 1993; Lander and Schork, 1994; Paterson *et al.*, 1988). The analyses of quantitative trait loci (QTL) segregating in hybrids of different organisms revealed that, in some cases, a small number of genetic loci have a large influence on the variance of each trait, (Flint and Mott, 2001).

QTL analysis looks for associations between the quantitative trait and marker alleles segregating in the population. It has two essential stages; the mapping of the markers and the association of the trait with the markers (Kearsey and Farquhar, 1998). Both of these stages require accurate data and must satisfy statistical analysis. The mapping of QTLs has been available since 1920s, based on polymorphic morphological markers (Mather, 1938), however the methods for detecting associations has been modified to handle hundreds of markers at the same time. Although slightly different algorithms can be used in the final stages to "smooth" the results to fit the multiple marker information, the maps produced are very similar (Lander *et al.*, 1987; Stam, 1993). However, the quality of the marker data from the segregating population is very important for the analyses.

The major difficulty in identifying QTLs is that a single QTL with a small effect might explain only a small proportion of the phenotypic variation and therefore the phenotype-genotype correlation will be low. However, since RFLP markers were first used in 1980s (Beckmann and Soller, 1983; Lander and Botstein, 1989), and later with different PCR based markers such as RAPDs, microsatellites (Tautz, 1989) and AFLPs (Vos *et al.*, 1995), and with the advantages of PCR, marker genotypes are cheaper, faster, and can provide a high density of polymorphic markers within small DNA fragments (Westman and Kresovich, 1997). The construction of linkage groups with larger densities of polymorphic markers makes it easier to locate QTLs more precisely by detecting recombinations between markers and QTLs. However, the analysis of QTLs has also been made easier with recently developed statistical analysis (Sen and Churchill, 2001; Seaton, 2002). In the early days, analysis of QTLs involved regression of phenotype data onto information from single markers. It now ranges from multiple (regression-based) interval mapping to likelihood-based Bayesian approaches (eg. Haley and Knott, 1992; Jansen, 1993; Jansen and

Stam, 1994; Zeng, 1994; Satagopan *et al*, 1996; Kao *et al*, 1999; Carlborg *et al*, 2000; Corander and Sillanpaa, 2002; Jansen *et al*, 2003), incorporated into several software packages (Goffinet and Gerber, 2000; Khatkar *et al*, 2004; Arcade *et al*, 2004; Sawkins *et al*, 2004).

As QTL mapping became easier, several quantitative trait genes were identified successfully. For example, the *fw2.2* locus was identified in hybrids between a large-fruited commercial tomato and a small-fruited wild-relative as an important QTL for fruit size and subsequently cloned by fine mapping (Frary *et al.*, 2000; Lippman and Tanksley, 2001; Cong *et al.*, 2002; Figure 1). Other fruit size related genes were also detected; however, *fw2.2* is a major QTL that accounts for as much as 30% of variance in fruit size (Cong *et al.*, 2002). The same mapping populations have been used to detect QTL for self pollinating (Georgiady *et al.*, 2002), and leaf dissection (Holtan and Hake, 2003) in tomato.

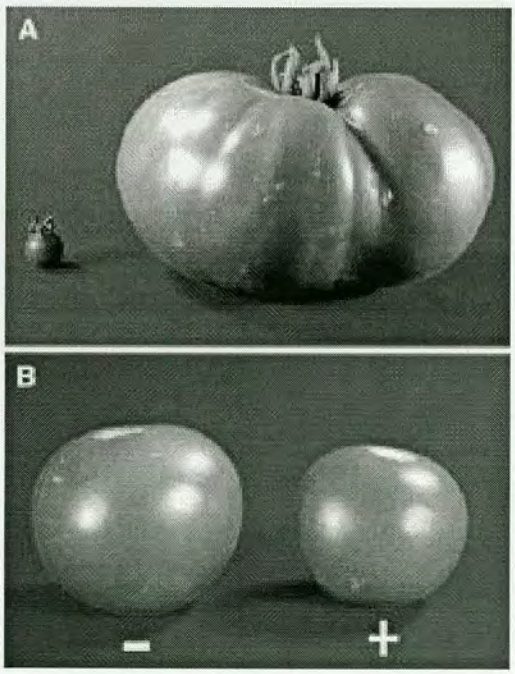



**Figure 1 (A) Tomato fruit size difference between wild species (*Solanum pimpinellifolium*) with small fruit and cultivar species (*S. esculentum* cv. Giant) with extremely large in size. (B) Phenotypic effect of the *fw2.2* transgene in the cultivar Mogeor** (Reproduced from Frary, 2000).

In examining evolution of apical dominance in maize (*Zea mays*), Doebley and Stec (1993) detected two QTL with major effects on plant and inflorescence architecture of maize and teosinte, the wild maize relative (Figure 1.1). One QTL mapped close to a locus known from the loss-of-function mutation *teosinte branched 1* (*tb1*) on chromosome 1 in

maize to be involved in branching and the gene was cloned by transposon tagging in maize (Doebley and Stec, 1993). Several similar studies have succeeded in detecting QTLs responsible for morphological differences in maize (Doebley and Stec, 1993; Lauter and Doebley, 2002; Hubbard *et al.*, 2002), as well as in nitrogen abundance (Coque and Gallais, 2006). More detailed studies were carried out to understand leaf evolution in *teosinte* (Lauter *et al.*, 2004; Bomblies and Doebley, 2006).

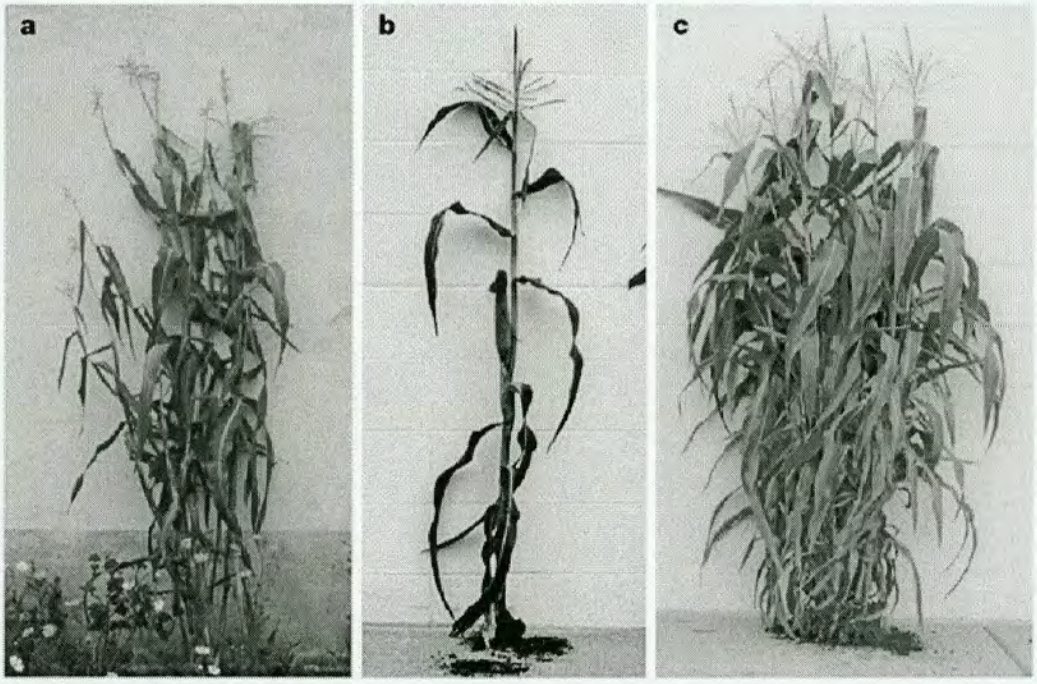



**Figure 1.1 The evolution of apical dominance in maize (*Zea mays*).** **a** | The maize crops that are cultivated today are probably a domesticated form of the wild Mexican grass, teosinte. Note the bushy form of the teosinte, *Zea mays* ssp. mexicana, shown here. **b** | The single-stalk branching pattern of wild-type maize. **c** | A maize plant that is mutant for the *teosinte branched 1 (tb1)* gene. The *tb1* locus is likely to have had an important role in the evolution of maize plant architecture (Reproduced from Mauricio, 2001).

*Arabidopsis thaliana* has been studied to identify QTLs for a number of traits, including morphology of shoots and roots and reproductive traits (Perez-Perez *et al.*, 2002; Juenger *et al.*, 2005; Fitz Gerald *et al.*, 2006; DeCook *et al.*, 2006) (Figure 1.2). Different QTLs affecting flowering time were also detected (El-Assal *et al.*, 2001; Koornneef *et al.*, 1998; El-Lithy *et al.*, 2006), as well as other quantitative differences such as the ability to adapt to environmental changes, water and anion content (Malmberg *et*

*al.*, 2005; Loudet *et al.*, 2003) as well as circadian clock period and trichome densities (Edwards *et al.*, 2005; Symonds *et al.*, 2005).

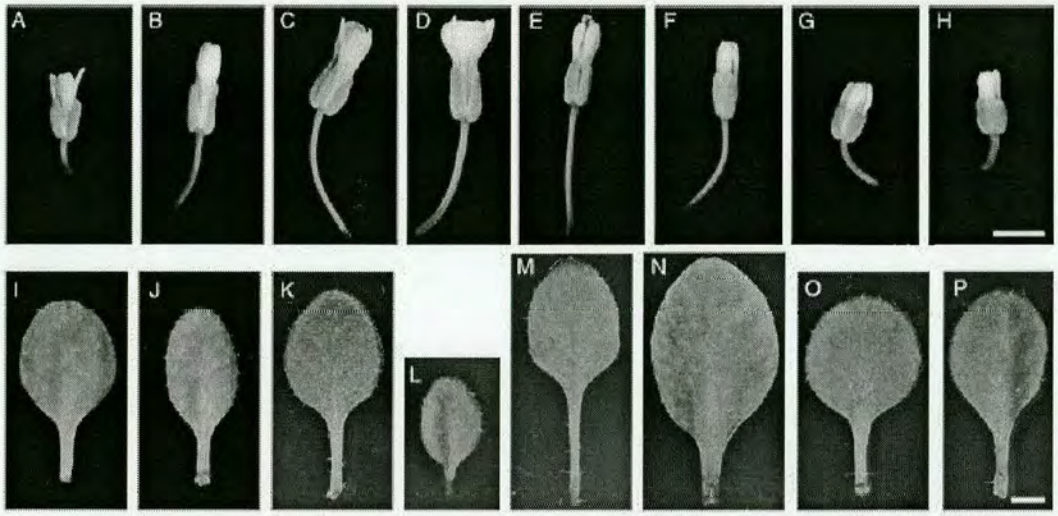



**Figure 1.2 Vegetative and reproductive differences of *Arabidopsis thaliana* lines. A-H:** show segregation for flower morphology and **I-P:** segregation for leaf morphology in hybrids between two ecotypes. Scale bars indicate 2mm. (Reproduced from Juenger *et al.*, 2001).

In tomato and maize the changes being analysed have been under strong human selection and domestication; it will therefore be interesting to extend these approaches to non-crop species. Although Arabidopsis is a non-crop species, it could be argued that because it is a single species the variation found within it is not typical of the differences involved in speciation. *Antirrhinum majus* is a model species that has not been subject to intense selection and offers the opportunities to analyse natural morphological variation between species as it can be crossed to wild species such as *Antirrhinum molle*.

## Plant Architecture and Morphological Evolution

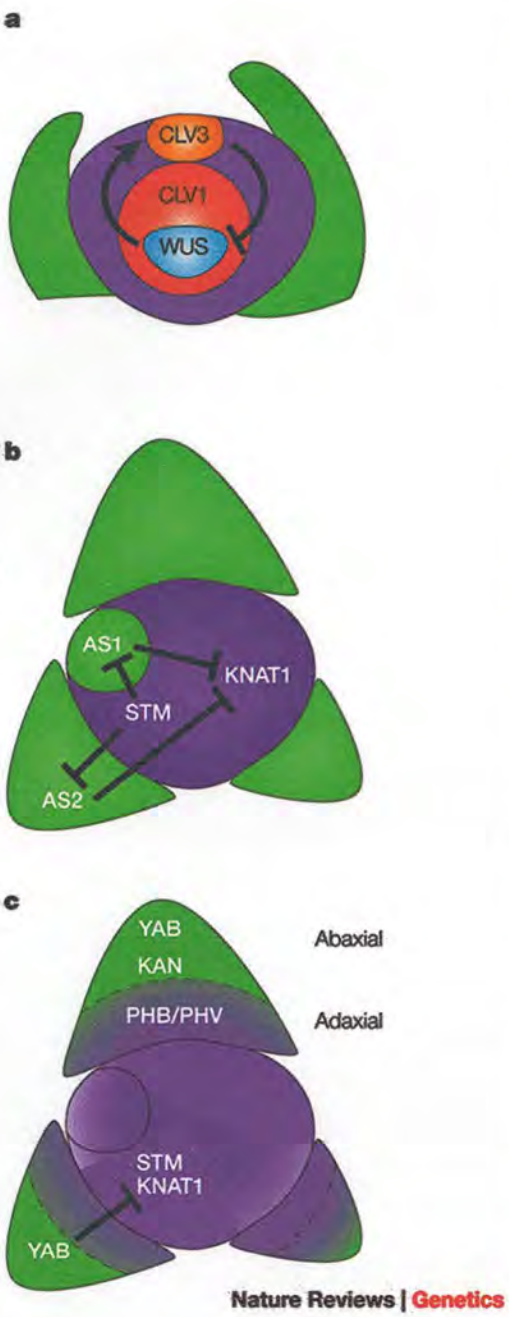
Plant architecture is complex and the result of many different genetic and environmental interactions. A better understanding of how genes control plant form can help modification of specific traits that are of relevance to agriculture and horticulture (Reinhardt and Kuhlemeier,

2002). During the past few years, studies with model plants such as *Antirrhinum majus* and *Arabidopsis thaliana*, and with some crops such as maize and tomato, have furthered our understanding of the genetic basis of plant architecture.

## • Leaf Architecture

Leaves (during the vegetative phase) and flowers (in the reproductive phase) are major elements of plant architecture. The shapes and sizes of leaves or of flowers vary widely between species. The fossil record indicates that primitive plants had branched stems with sporangia but no leaves suggesting that leaves are a relatively recent innovation in plants (Kenrick and Crane, 1997; Gifford and Foster, 1989).

Different developmental processes are required for leaf formation. Firstly, leaf initials are specified at specific sites in the shoot apical meristem (SAM). Secondly, cells in a leaf primordium are directed to different developmental identities. Finally, the leaf grows to its final size and shape through cell division and cell expansion (Figure 1.3, Hay *et al.*, 2004).




**Meristem signalling**

**a:** shows a longitudinal view of a shoot apex. *CLAVATA 3* (*CLV3*) is expressed in a central domain of the SAM and is thought to act as a ligand for the CLV1 receptor kinase and to function as part of a protein complex. This complex limits the activity of the homeodomain transcription factor WUSCHEL (WUS), which is required for meristem activity and induces meristem fate when mis-expressed in leaves. WUS activity, in turn, activates CLV3 activity. So, WUS promotes the activity of its own repressor and establishes a feedback loop that is predicted to maintain an equilibrium state and, consequently, a relatively constant cell number in the SAM.

**Meristem–leaf signalling**

**b:** shows a transverse view of a shoot apex. *SHOOTMERISTEMLESS* (*STM*) encodes a class I KNOX homeodomain transcription factor that is expressed throughout the SAM (purple) but is absent from leaf founder cells (indicated as a green domain in the SAM). *ASYMMETRIC LEAVES1* (*AS1*) encodes a MYB transcription factor and *AS2* is a member of the LATERAL ORGAN BOUNDARIES family of putative transcription factors. Expression of these two genes is excluded from the SAM and restricted to leaf primordia (green). Genetic analyses indicate that *STM* negatively regulates *AS1* and *AS2* function in the SAM and downregulation of *STM* in leaves allows *AS1* and *AS2* expression. *AS1* and *AS2*, in turn, negatively regulate other class I *KNOX* genes so that *KNAT1*, *KNAT2* and *KNAT6* are ectopically expressed in the leaves of *as1* and *as2* mutants. The additional loss of *KNAT1* function in *stm;as1* double mutants results in loss of a SAM, indicating that *KNAT1* functions redundantly with *STM* in maintaining a SAM.

**Leaf–meristem signalling**

**c:** shows a transverse view of a shoot apex. *PHABULOSA* (*PHB*) and *PHAVOLUTA* (*PHV*) encode class III homeodomain zipper (HD-ZIP) transcription factors that are expressed throughout the SAM, with high expression in rays extending from the SAM to the youngest leaf primordia, and in the adaxial domain of older leaf primordia (purple). *YABBY* (*YAB*) and *KANADI* (*KAN*) gene families encode putative transcription factors: YAB proteins contain zinc finger and high mobility group (HMG) domains, and *KAN* genes belong to a larger gene family of transcriptional regulators that contains a group domain. They are expressed in the abaxial domain of leaf primordia (green) and *YAB* members repress *STM* and *KNAT1* expression in the leaf.

**Figure 1.3 Signalling in and between meristem and leaves** (Reproduced from Tsiantis & Hay. 2003).

The role of *knotted1-like homeobox (KNOX)* genes in leaf development has been widely studied. *KNOX* genes belong to a plant-specific clade of the Three Amino acid Loop Extension (TALE) superclass of homeobox genes (Piazza, Jasinski and Tsiantis, 2005). The first plant homeobox gene, *knotted1* (*kn1*), was discovered from gain-of-function mutations affecting the maize leaf (Vollbrecht *et al.*, 1991) that caused *kn1* misexpression in developing leaves and the formation of epidermal outgrowths ('knots'). The expression of *kn1* and other *KNOX* genes was initially found to be restricted to the SAM where they are required to maintain SAM activity (Long *et al.*, 1996; Vollbrecht *et al.*, 2000; Byrne, 2002). Down-regulation of *KNOX* expression in leaf initial cells provided one of the earliest indications of leaf fate. Ectopic *KNOX* expression in leaves, resulting either from gain-of-function mutations in *KNOX* genes or loss-of-function mutations in their repressors (e.g. member of the *phantastic* (*phan*)-like family of MYB genes) causes a range of developmental defects. These include induction of ectopic meristem development in Arabidopsis and tobacco (Tattersall *et al.*, 2005; Byrne *et al.*, 2000; Ori *et al.*, 2000), formation of sheath-like tissues in the maize leaf blade due to mis-expression of the *KNOX* gene *Liguless3* (Muehlbauer *et al.*, 1997); defects in organ size, shape and dorsiventral polarity (see below),and production of new leaf axes (McHale and Koning, 2004; Waites and Hudson, 1995). Golz *et al.* have studied the developmental the morphogenetic potential of KNOX proteins outside the leaf, showing that ectopic expression of the *KNOX* genes *Hirzina* (*Hirz*) and *Invaginata* (*Ina*) mutations can cause the formation of a new floral structure in *Antirrhinum* (Golz *et al.*, 2002).

However, these initial studies involved species with simple leaves. Studies of the development of complex leaves have been carried out mostly in tomato and pea (reviewed in Sinha, 1999). In tomato, the leaf is compound and, after initial loss of expression before primordium initiation, developing primordia express *KNOX* genes (Koltai and Brid, 2000). Over-expression of *KNOX* genes in tomato results in super compound leaves (e.g. Hareven *et al.*, 1996). Similarly, relatives of Arabidopsis in the *Brassicaceae* with compound leaves can also express *KNOX* genes (Champagne and Sinha, 2004).

Studies on the role of hormones found that KNOX regulates synthesis of the hormones gibberellic acid (GA) and cytokinin. When the *KNOX* gene, *Nicotiana tabacum Homeobox15* (*NTH15*) was mis-expressed in tobacco leaves,

application of GA could restore the defects in leaf shape (Tamaoki *et al.*, 1997), suggesting that KNOX acted to suppress GA biosynthesis. NTH15 was later found to suppress expression of the gene encoding the GA-biosynthetic enzyme GA-20 oxidase (Sakamoto *et al.*, 2001), and regulation was likely to be direct because NTH15 could bind directly to the GA-20 oxidase gene promoter (Hay *et al.*, 2002; Sakamoto *et al.*, 2001). Application of GA was also found to reduce the effects of ectopic *KNOX* expression in *Arabidopsis* leaves at early stages of development (Piazza, Jasinski and Tsiantis, 2005; Hay *et al.*, 2002), as was the presence of the *spindly* mutation that causes constitutive GA signalling. This suggested that KNOX expression in the shoot apical meristem maintained the stem cell population of the meristem by maintaining low GA levels. In tomato, over-expression of KNOX results in the formation of more complex leaves, however GA acts to restrain the effects of *KNOX* over-expression (Hay *et al.*, 2002), suggesting that the role of *KNOX* genes in promoting compound leaf development are partly mediated through reduction of GA activity. Similarly *KNOX* mis-expression was found to increase expression of genes encoding cytokinin biosynthetic genes (Jasinski *et al.*, 2005; Yanai *et al.*, 2005), consistent with a normal role of *KNOX* genes in maintaining low GA and high CK in the shoot apical meristem and high GA and low CK contributing to the effects of *KNOX* mis-expression in leaves (Figure 1.4).

**Figure 1.4 *KNOX* gene expression and leaf form** (Reproduced from Tsiantis & Hay. 2003).)

The margin of a simple leaf can be smooth (panel **a**, left), serrated (not shown) or separated into lobes by sinuses that extend one quarter or more of the distance to the midvein (panel **b**, left). A dissected leaf (panel **c**, left) has a margin that is divided into individual units called leaflets. Regions of organogenic activity at the leaf margin have been termed 'blastozones'; these regions can develop as leaflets, lobes or serrations depending on their organogenic potential. Overexpression of *KNOX* genes in *Arabidopsis* results in delayed growth in the sinuses between margin serrations, so that deep lobes develop in the normal position of serrations. Persistence of KNOX expression, therefore, would seem to confer prolonged organogenic potential to regions at the leaf margin. Indeed, KNOX expression persists in the developing primordia of many dissected leaf species.

The figure shows a hypothetical model of how recruitment of KNOX function into leaves results in dissected form. In *Arabidopsis*, the genes *SHOOTMERISTEMLESS* (*STM*), *KNAT1* and *UNUSUAL FLORAL ORGANS* (*UFO*) are expressed in the shoot apical meristem (SAM; purple in panel **a**), whereas *ASYMMETRIC LEAVES1* (*AS1*), *AS2* and the gibberellin biosynthetic gene *GA20ox1* are expressed in the leaves (green). *STM* negatively regulates *AS1*, *AS2* and *GA20ox1* and positively regulates *UFO*, whereas *AS1* and *AS2* negatively regulate *KNAT1*. These interactions confine indeterminacy factors to the SAM and determinacy factors to the leaf, resulting in a simple leaf form. The ectopic expression of *KNAT1* or *STM* in *Arabidopsis* (panel **b**, middle), driven by the CAMV 35S PROMOTER, negatively regulates *GA20ox1* in leaves, contributing to a lobed leaf form. Ectopic expression of *UFO* also results in lobed leaves (panel **b**, right). So, the presence of indeterminate factors in the leaf and/or the repression of determinacy factors produce a lobed leaf form. This model proposes that leaf expression of *KNOX* genes in certain species (such as tomato; panel **c**, middle) results in the repression (for example, of *GA20ox1*) and possible activation of KNOX targets in leaves (for example, of *UFO*) and thereby generates a dissected leaf form. In pea (panel **c**, right), KNOX-independent recruitment of indeterminacy factors (such as *UFO*) for function in the leaf leads to the dissected leaf form.

Note that 'dissected' leaves are sometimes referred to as being 'compound' – because of the presence of leaflets and some controversy surrounds which is the more appropriate definition. Some proponents of the former term object to the latter as they believe that it negates the fact that both types of organ are equivalent structures that are delimited from the SAM in a similar manner. By contrast, other workers maintain that the term 'compound' is necessary to differentiate between the deployment of distinct developmental programmes in the development of the two structures.

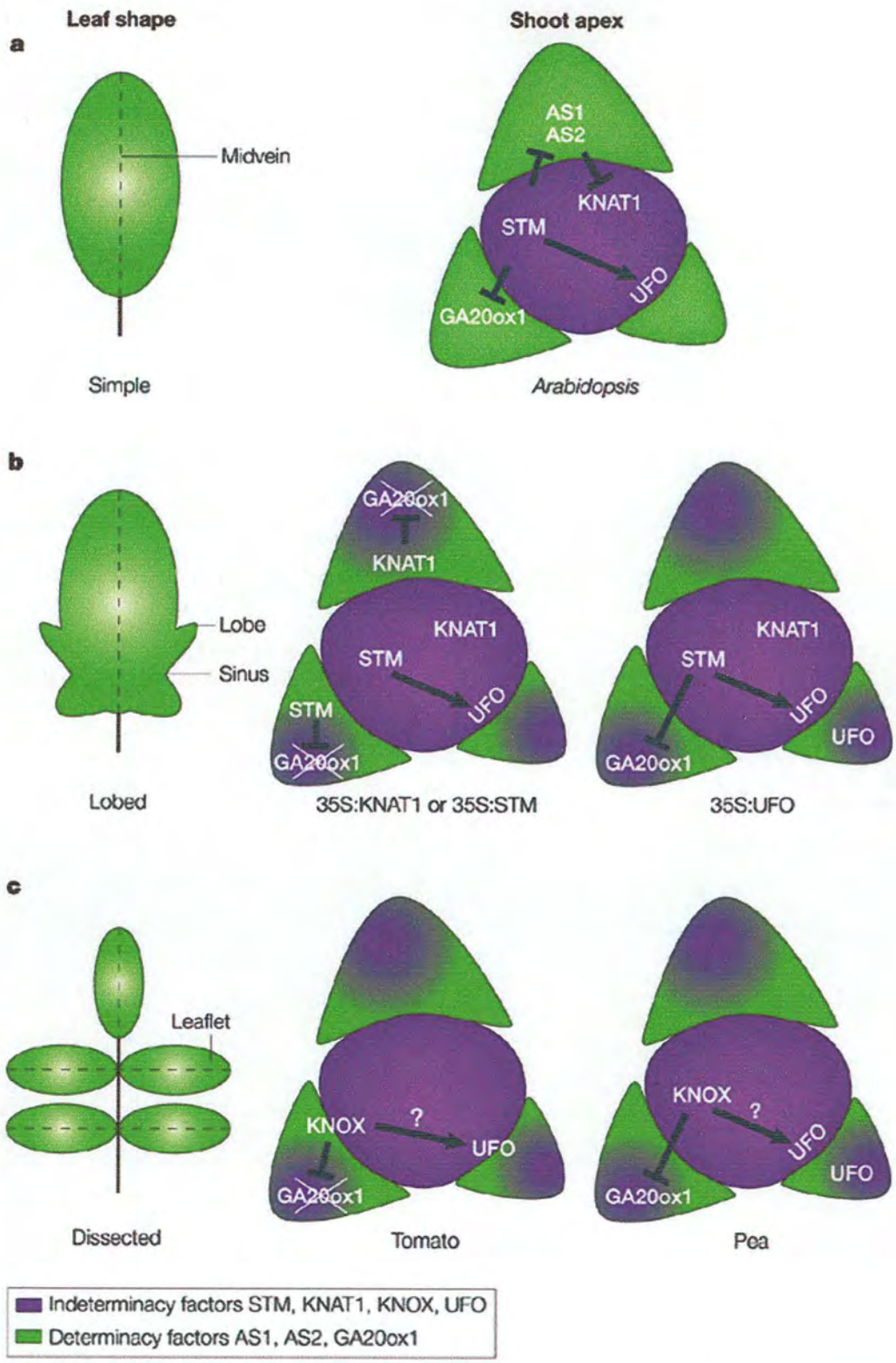



**Figure 1.4 *KNOX* gene expression and leaf form** (Reproduced from Tsiantis & Hay. 2003).)

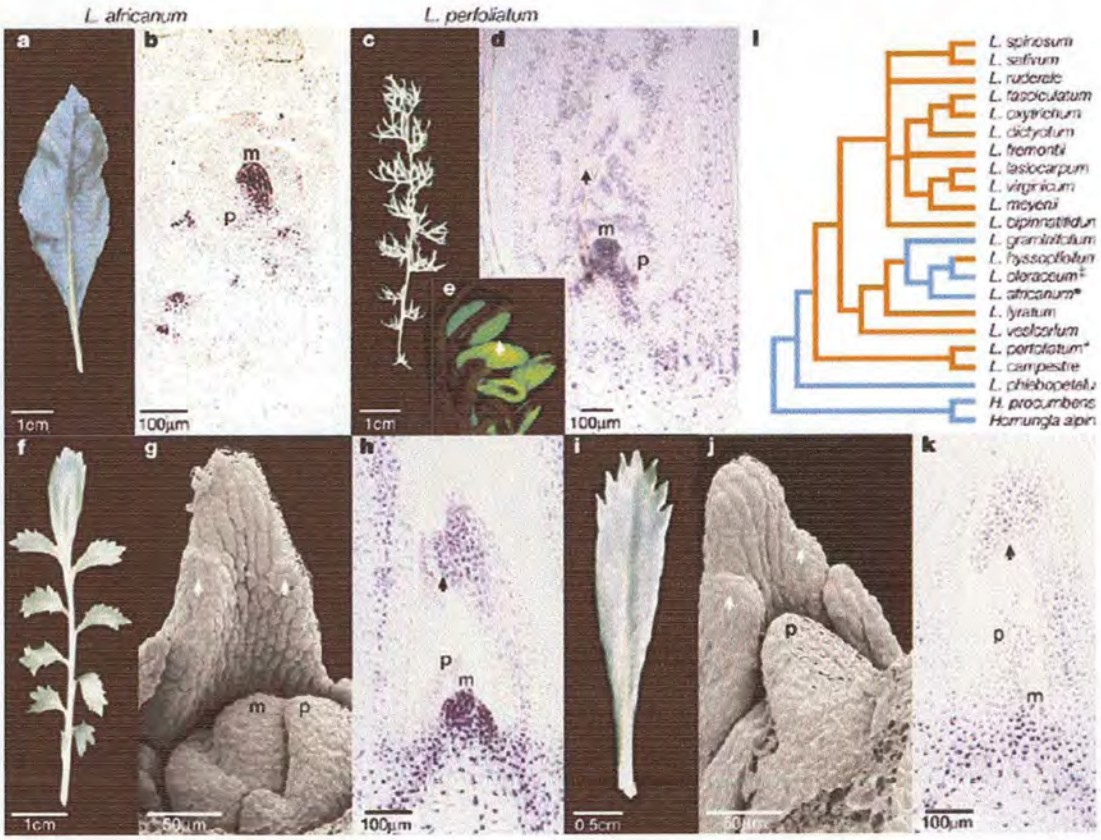



**Figure 1.5 Consistent correlation between leaf form and KNOX gene expression in Brassicaceae.** The final leaf forms of four different *Lepidium* species are shown with *KNOX* gene and KNOX protein expression. The final leaf form is shown in **a**, **c**, **f** and **i**. Protein expression is shown in **b**, **d**, **h** and **k**. Panels **g** and **j** show scanning electron micrographs of *L. hyssopifolium* and *L. oleraceum* developing leaves, respectively. The result shows that KNOX is expressed in the SAM (m) of all species and is absent from incipient primordia (p); however, it is present on the dissected leaf (arrow) during the leaflets developing, which is consistent with the model that KNOX gene expression in leaves increases organogenic potential. In panel **e**, a whole-mount reverse transcription-polymerase chain reaction *in situ* hybridization shows expression of the *KNOX* gene *STM1* in developing dissected leaflets. Panel **l** shows phylogenetic patterns of leaf evolution in genus *Lepidium*. Colours of the branches indicate ancestors with simple (blue) or dissected (orange) leaves, showing that there have been multiple switches between simple and dissected leaf form in the evolution of *Lepidium*. (Figure reproduced from Tsiantis and Hay, 2003).

Other molecular analyses also showed that KNOX-independent mechanisms might also contribute to complex leaf morphology in legumes (Hofer, 2001). Hofer *et al.* (1997) first showed that loss-of-function of *UNIFOLIATA* (*UNI*), a pea gene encoding a transcription factor similar to *LEAFY* (*LFY*) in *Arabidopsis* (Weigel, 1992) and *FLORICULA* (*FLO*) in *Antirrhinum* (Coen *et al.*, 1990), changes pea leaves from dissected to simple form. In contrast, pea leaves do not normally express *KNOX* genes and alterations in *KNOX* expression do not affect development of the pea compound leaf (Gourlay *et al.*, 2000). This suggests that compound leaves of legumes might have evolved independently from compound leaves in *Brassicacae* and *Solanaceae*.

One of the most obvious changes following initiation of a leaf primordium is elaboration of polarity within the leaf. A prototypical leaf has three axes: the proximodistal axis (tip-base), the dorsiventral axis (adaxial-abaxial) and the lateral (left-right) (Reinhardt and Kuhlemeier, 2002). Dorsiventral polarity is often associated with specialisation of tissue, for example with an upper (adaxial) side specialized for light capture, and an lower (abaxial) side specialized for gas exchanges.

Classical experiments show that radially symmetric leaves can result from separation of incipient leaf primordia from the shoot apical meristem, indicating that the meristems are essential in establishing dorsiventrality (Sussex, 1955). Genetic analysis has identified several mutants in which the leaves are radially symmetric (reviewed in Bowman *et al.*, 2002). These findings have led to that view that signals from the meristem initiate dorsal identity in the adaxial side of the primordium closer to the meristems and presumably that the signals are not able to reach the abaxial primordium. The dorsal and ventral identities are then restricted by mutual repression to establish two separate domains. The interaction between the domains leads to outgrowth of the blade (Bowman *et al.*, 2002).

The genetic control of adaxial-abaxial polarity was first studied in *Antirrhinum majus* (Waites and Hudson, 1995). The *Antirrhinum phantastica* (*phan*) mutant has radially symmetrical leaves and was explained by abaxialization of dorsal cells (Waites and Hudson, 1995). Analysis of *phan* mutations indicated that cells destined to be abaxial and adaxial are required for normal lamina outgrowth; suggesting that the signalling between two cell types is required to induce growth (Sussex, 1954; Sussex,

1955) and that establishment of polarity is required for lamina development (Bowman, Eshed and Baum, 2002). MicroRNAs (miRNAs) appear to be important in the early stages of leaf polarisation through regulation of different types of HD-ZIP III transcription factors needed for adaxial identity in leaf and vasculature (Carrington and Ambros, 2003; Hay *et al.*, 2004).

MicroRNAs are single-stranded RNA molecules of around 21-24 nucleotides (nt) that were first found in nematodes (Lee, Feinbaum and Ambros, 1993), and now are known in both metazoans and plants (Carrington and Ambros, 2003; Bartel, 2004). The elucidation of miRNAs roles in plant leaf development has involved analysing genes required for miRNA formation or activity, and identification of miRNA target genes (Carrington and Ambros, 2003). In *Arabidopsis*, loss-of-function mutants with reduced DICER-LIKE1 (DCL1) activity, needed for miRNA processing, affect embryogenesis and both vegetative and reproductive development (Schauer *et al.*, 2002), suggesting that miRNAs act negatively to control meristem cell identity, organ polarity, and other developmental processes (Llave *et al.*, 2002; Reinhart *et al.*, 2002; Park *et al.*, 2002). In angiosperms, organ axis specification and vascular development involve regulation of HD-ZIP III genes by miR165/miR166 (Juarez *et al.*, 2004; McHale and Koning, 2004; Kidner and Martienssen, 2004; Mallory *et al.*, 2004; Emery *et al.*, 2003).

The HD-ZIP III genes such as *PHABULOSA* (*PHB*) and *PHAVOLUTA* (*PHV*), and their regulation by miRNA, are highly conserved within green plants (Floyd and Bowman, 2004). *PHV* and *PHB* control the initiation of adaxial (upper)-abaxial (lower) polarity, specialized leaf structure and function (Carrington and Ambros, 2003). Analysis of *Nicotiana sylvestris PHAVOLUTA* (*NsPHAV*) mutations suggests they may have a general role in the control of indeterminate cell fate (McHale and Koning, 2004).

*PHV* and *PHB* are normally expressed near the shoot meristem to specify adaxial organ cell fate while cells further from the meristem do not express HD-ZIP III activity, due to the presence of the miR165/166, resulting in their development as abaxial cells (McConnell *et al.*, 2001). Mutations in HD-ZIP III genes that alter the binding site for miR165/166 result in dominant *phv* and *phb* alleles that are expressed abnormally in abaxial cells, converting their identity from abaxial to adaxial (Rhoades *et al.*, 2002; Tang *et al.*, 2003). It has been suggested that the miR165/166 might be part of the signal from the shoot apical meristem that orients organ

polarity with the central-peripheral axis of the SAM (Kidner and Martienssen, 2004), although this does not explain why a miRNA from the meristem might repress HD-ZIP III expression abaxially but not in the adaxial part of organ primordia which it would have to pass through.

The *YABBY* family of transcription factor genes was found to be involved in abaxial identity and growth in leaves and flowers in *Arabidopsis*. *YABBY* genes are expressed in abaxial regions of lateral organs like leaves and flowers and are required redundantly for abaxial cell identity (Siegfried *et al.*, 1999). The ectopic expression of *FILAMENTOUS FLOWER* (*FIL*) and *YABBY3* (*YAB3*) genes can specify some abaxial characteristics in adaxial cells in the *Arabidposis* leaf (Siegfried *et al.*, 1999).

The *KANADI* (*KAN*) gene family was also shown to specify abaxial identity in leaf primordia (Kerstetter et al., 2001), because *kan* mutants were found to have adaxial characteristics in the abaxial side of leaves. Consistent with this, the *KAN* gene is expressed in the abaxial side of cotyledons in embryos and on the abaxial side of leaf primordia and primordia of floral organs and can cause abaxialisation of cells when expressed adaxially (Eshed *et al.*, 1999).

However, although it is clear that these genes can repress each other's expression, the sequence in which they normally do this is not clear. Although it is often assumed that asymmetric HD-ZIP III gene expression is established first and this leads to repression of *KANADI* and *YABBIES*, the ability of *KANADI* and *YABBY* genes to cause ectopic abaxial identity when expressed ectopically suggests that it might be the other way round.

After leaves are initiated from the SAM, cell growth and division occurs throughout the primordium, then cell division ceases from the tip of the leaf down to the base. The patterns of regional growth leading to the final shape of a leaf have been studied by following the fate of marked clones of cells from several species (e.g. Poethig and Sussex, 1985; Dolan and Poethig, 1998). Regulation of this growth is known to involve several regulatory genes, although the relationship between the processes they control is often not clear.

Examining the *aintegumenta* (*ant*) mutant of *Arabidopsis* Mizukami & Fischer (2000) revealed that *ant* mutant leaves were smaller than wild-type and that overexpression of *ANT*, an AP2-like transcription factor, increased

leaf size by prolonging the period of cell division. This suggests that *ANT* regulates cell proliferation and organ growth by maintaining the meristematic competence of cells during organogenesis (Mizukami and Fischer, 2000; Nole-Wilson and Krizek, 2006). Similar effects on leaf size were found on over-expression of an inhibitor of cyclin-dependent kinase (ICK1; Wang *et al.*, 2000) or disruption of a GTP-binding protein, *GPA1* implicated in cell-cycle control (Ullah *et al.*, 2001). All result in leaves of reduced size, as compared to wild-type, and the number of cells in the lamina decreases while the volume of leaf cells increases, suggesting that increased cell expansion might partly compensate for reduced cell numbers.

Another gene, *ARGOS*, which is highly induced by auxin, was found to be involved in organ size control in *Arabidopsis*. Analysis of the *argos* mutant showed that aerial organs had more cells than wild type and were enlarged. Ectopic expression of *ARGOS* prolonged the expression of *ANT* and the mitotic cyclin *CycD3;1*. Moreover, organ enlargement in plants overexpressing *ARGOS* could be blocked by the loss of *ANT* function, implying that *ARGOS* functions upstream of *ANT* to affect the meristematic competence of organ cells. The induction of *ARGOS* by auxin is reduced or lost in *auxin-resistant1* (*axr1*) mutants that have partially lost auxin-induced growth responses, and overexpression of *ARGOS* can partially restore growth in *axr1* organ development. These results suggest that *ARGOS* may transduce auxin signals downstream of *AXR1* to regulate cell proliferation and organ growth through *ANT* during organogenesis (Hu *et al.*, 2003).

Another family of transcription factor genes that might be involved in determining meristematic competence is TCP. The *cincinata* (*cin*) mutant of *Antirrhinum*, which lacks activity of the *CIN* TCP gene, has leaves that have a larger area than wild-type and are not flat because of increased growth and cell numbers in marginal regions. This suggests that *CIN* acts to repress cell division in leaf margins, consistent with its expression in cells as they cease division (Nath *et al.*, 2003). In *Arabidopsis* related *TCP* genes appear to have a similar role and are regulated by a miRNA encoded by the *JAW* locus. Increased expression of *JAW* miRNA leads to down-regulation of *TCP* genes and a phenotype similar to that of *cin* in Antirrhinum (Palatnik *et al.*, 2003). Recently, another class of TCP transcription factors has been found to promote co-ordinate expression of a mitotic cyclin, required for cell division, and genes involved in

cell growth (Li *et al.*, 2005). This lead to the suggestion that such positively acting TCP transcription factors compete with TCP transcription factors that promote differentiation and cell expansion in the regulation of organ growth.

A RING-finger protein, BIG BROTHER (BB) has been identified recently as a novel repressor of petal growth in *Arabidopsis*. Petal size is sensitive to the level of BB expression, which affects the duration of cell division and growth, suggesting that growth stimulators might act via degradation of BB (Disch *et al.*, 2006). Arabidopsis plants lacking activity of the receptor kinase *ERECTA* (*ER*) have smaller leaves that are rounder and have shorter petioles than wild-type (Torii *et al.*, 1996). This suggests that *ER* is involved in transducing a signal that promotes organ growth, although the identity of the ER ligand is not known.

## • Branching and Plant Architecture

Flowering can affect plant architecture in many ways. In some plant such as *Antirrhinum*, with decussate phyllotaxis, it is associated with a transition to spiral phyllotaxis (Carpenter *et al.*, 1995). In many plants such as *Antirrhinum* and *Arabidopsis*, the shoot apical meristem of the main shoot is indeterminate; it is active during the whole plant life, producing leaves during the vegetative phase and then flowers later in the reproductive phase. This growth behaviour is referred to as monopodial (Schmitz and Theres, 1999). In contrast to the situation in monopodial plants, in some plants like tomato (*Solanaceae* family), the SAM is determinate; the main shoot stops growth after a single inflorescence is produced, however, growth continues from the lateral meristems. Such growth behaviour is referred to as sympodial (Schmitz and Theres, 1999).

Mutation analysis of the *LFY* gene in *Arabidopsis* and its orthologue, *FLORICAULA* (*FLO*), in *Antirrhinum* show that they are involved in transforming flowers into indeterminate axillary branches (Coen *et al.*, 1990; Weigel *et al.*, 1992). Thus, *FLO* and *LFY* can switch the developmental phase from indeterminate axillary meristems into determinate floral meristems. These findings support the view that flowers represent axillary shoots (Coen and Nugent, 1994). Opposite developmental effects were caused by mutations of *CENTRORADIALIS* (*CEN*) of *Antirrhinum* and *TERMINAL FLOWER1 (TFL1)* of *Arabidopsis* in which the SAM terminates in

flower formation; thus growth is switched from indeterminate to determinate (Bradley *et al.*, 1996, 1997). Conversely, *CEN* over-expression in tobacco (a determinate plant) extends the vegetative growth phase (Amaya *et al.*, 1999).

Most plants also produce lateral shoots from axillary meristems that are initiated from the axils of leaves. The lateral shoots contribute to overall plant architecture; therefore, the branching pattern partly reflects the phyllotactic pattern of the main shoot axis. In most plants, apical dominance is applied and the shoot tip restrains the growth of axillary meristems (Davies, 1995). When apical dominance is increased, branching is reduced, one of the major traits selected during the domestication of cultivated maize from its wild relative, teosinte. The *teosinte branched 1 (tb1)* gene was found have an important role controlling apical dominance (restraining branching) in maize (Doebley and Stec, 1993). *Tb1* retains apical dominance in maize, loss-of-function *tb1* results in maize with branching phenotypes like teosinte (Doebley and Stec, 1993).

In tomato, increased branching requires more manual pruning for high yields. The *lateral suppressor (ls)* mutants, which have no lateral branches because vegetative axillary meristems are suppressed (Schumacher *et al.*, 1999), are therefore of horticultural interest. Four different *MAX* genes were found in *Arabidopsis*, *MAX1*, *MAX3*, and *MAX4* are involved in hormone synthesis, and *MAX2* acts in its perception (Booker *et al.*, 2005). Analysis of *Arabidopsis MAX4* mutant shows increased lateral branches and resistance to auxin function. Similar result have been found in pea for the *rsms1* mutant (Sorefan *et al.*, 2003).

## • Control of Epidermal Cell Differences

The plant epidermis consists of different types of cells and their development involves numerous protein and gene interactions. One of the best studied examples is the formation of trichomes, which are each initiated from a single cell and result in single-cellular or multi-cellular structure (Werker, 2000). Different types of trichomes have been identified which can appear together in the same plant or in the same tissue of the plant. Generally, non-glandular trichomes are considered to protect plants from UV light and insects (Werker, 2000; Johnson, 1975; Mauricio and Rausher, 1997) and glandular trichomes to

protect plants from pathogens (Werker, 2000). Development of trichomes has been well studied in *Arabidopsis* (Hülskamp, 2004; Schellmann, *et al.*, 2002; Marks and Esch, 2003; Larkin, *et al.*, 1996), in which trichomes are single celled, non-glandular and usually have three branches. These are different to the trichomes of *Antirrhinum* and Solanaceous species, which are usually multi-cellular and can be glandular, and their formation appears to involve different transcriptional regulators (Serna & Martin, 2006).

A group of genes, including *GLABRA1* (*GL1*), *GLABRA2 (GL2)*, *GLABRA3* (*GL3*), and *TRANSPARENT TESTA GLABRA1* (*TTG1*), were found that are required for *Arabidopsis* trichome initiation and to be expressed in trichome precursor cells (Hülskamp *et al.*, 1999; Payne *et al.*, 2000). *GL1* encodes an R2R3 MYB transcriptional factor with two MYB repeats that comprise its DNA-binding domain (Oppenheimer *et al.*, 1991). Strong *GL1* mutants produce no trichomes indicating that *GL1* is required to specify trichome fate (Marks and Feldmann, 1989; Karkin *et al.*, 1993). Another R2R3 MYB transcriptional factor, *AtMYB23*, also shows functions partly similar to *GL1* (Kirik *et al.*, 2001; Kirik *et al.*, 2005). In yeast 2-hybrid, AtMYB23 interacts with basic-helix-loop-helix (bHLH) protein ENHANCER OF GLABRA3 (EGL3) (Zimmermann, et al., 2004), and the MYB domain of GL1 physically interacts with N-terminal domain of the bHLH protein GLABRA3 (GL3) and EGL3, which also promote trichome development (Payne *et al.*, 2000; Zhang *et al.*, 2003). *ttg1* mutants result in several pleiotropic defects including no trichome formation and the WD-40 TTG1 protein is thought to be part of the GL1-GL3 complex (Payne *et al.*, 2000). GL1, GL3 and TTG1, and their related proteins, are therefore thought to form a transcription factor complex that activates *GL2* expression, which is necessary and sufficient for trichome fate, in trichome precursor cells. The complex is also thought to contribute to the mechanism involved in spacing of trichomes because the complex promotes expression of the genes that encode it in trichome precursor cells and also activates expression of two short R2-R3 MYB proteins - TRYPTICHON and CAPRICE - that are proposed to move to neighboring cells where they inhibit expression of the complex and so inhibit trichome fate (Schnittger *et al.*, 1999).

Once trichome fate is specified growth and branching of the trichome depends on activity of a number of genes. Some of these are probably involved in regulating the actin cytoskeleton and others in control of

endoreduplication - DNA synthesis without cell division - which is necessary for trichome branching (Schnittger and Hülskamp, 2002).

Expression of a mitotic cyclin or loss of *SIAMESE* activity in Arabidopsis trichomes can cause formation of multi-cellular trichomes, leading to the suggestion that the unicellular trichomes of Arabidopsis evolved from multicellular trichomes (Schnittger and Hülskamp, 2002; Walker *et al.*, 2000). However none of the genes known to be involved in trichome development in Arabidopsis can be detected in the Antirrhinum EST collection (www.antirrhinum.net) or have been isolated from library screens (Beverley Glover, personal communication), suggesting that the multicellular trichomes of Antirrhinum might have evolved independently from those of Arabidopsis.

In *Antirrhinum majus*, *MIXTA* - an R2R3 MYB transcription factor only distantly related to GL1 - affects the polarity of petal epidermal cells (Noda *et al.*, 1994; Glover *et al.*, 1998; Martin *et al.*, 2002). *mixta* mutants have flat epidermal cells rather than conical cells in their dorsal (inner) petal epidermis (Glover, 1998). In *Antirrhinum*, *MIXTA* is normally only expressed in dorsal petal epidermal cells. When overexpressed in other parts of the tobacco plant, *MIXTA* induces the formation of multi-cellular trichomes and conical epidermal cells, suggesting that MIXTA-like proteins control both conical cell and multi-cellular trichome formation (Glover *et al.*, 1998; Payne *et al.*, 1999). In support of this, the *MIXTA-like1* gene is expressed in both conical epidermal cells in part of the *Antirrhinum majus* petal and in developing trichomes and its over expression in tobacco can induce multicellular trichome formation (Perez-Rodriguez *et al.*, 2005). Expression of a MIXTA-like MYB protein in Solanaceous species is also involved in trichome formation in anthers (Glover, 2004). This evidence supports a role of *MIXTA*-like genes in multicellular trichome formation in *Antirrhinum* and Solanaceae.

## • Control of Anthocyanin Biosynthesis in the Antirrhinum Flower

Anthocyanin biosynthesis in *Antirrhinum* flowers requires the activity of several genes encoding enzymes of the anthocyanin biosynthetic pathway (Martin *et al.*, 1991). Their expression is controlled by several other genes, among which a small family of MYB-Regulatory Genes, *Rosea1* (*Ros1*), *Rosea2* (*Ros2*), and *Venosa* (*Ve*), which are important for evolution of flower

pigmentation patterns (Schwinn *et al.*, 2006; Stubbe 1966). Analysis of *Rosea* and *Venosa* mutants show that these genes control the intensity and pattern of anthocyanin pigmentation in flowers (Schwinn *et al.*, 2006). Although these regulators are similar in structure, the effects of the genes encoding the enzymes of anthocyanin biosynthesis are different. *Rosea*$^{+}$ (Wild-type) flowers are almost completely red with anthocyanin apart from the central region of the ventral and lateral petals which are yellow due to the accumulation of aurone in the absence of anthocyanin. Anthocyanin also accumulates in the vegetative tissues such as leaves and stems. The *rosea* mutant has paler floral pigmentation compared to wild type. *Venosa*$^{+}$ shows strong red pigmentation in epidermal cells overlying petal veins in a *rosea* mutant background, suggesting that *Venosa* inhibits anthocyanin expression in the remainder of the flower (Schwinn *et al.*, 2006).

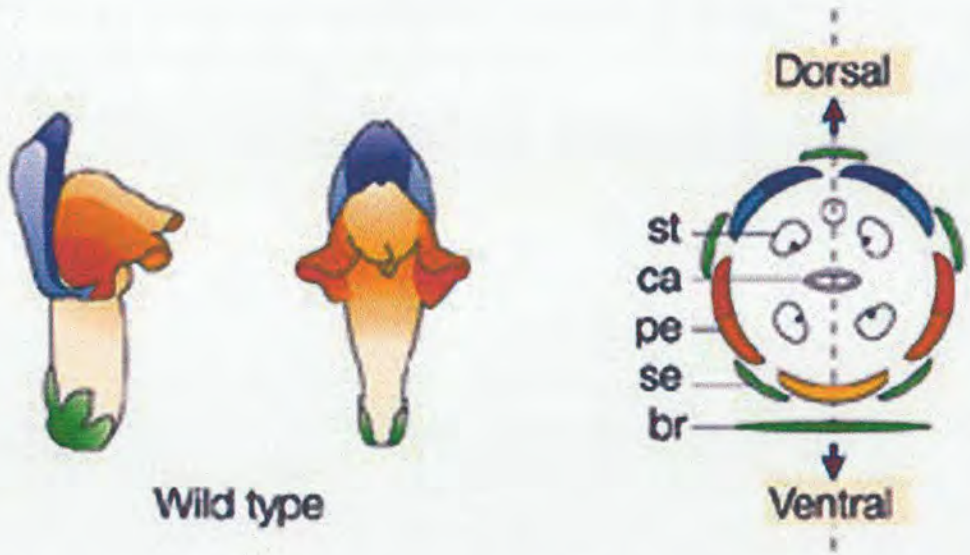



**Figure 1.6 Diagram of an Antirrhinum flower.** The wild type *Antirrhinum* flower is shown in lateral (left) and ventral (centre) views. The floral diagrams (right) represent the organization of the floral organs: bract (br), carpel (ca), petals (pe), sepals (se) and stamens (st). The corolla of wild-type *Antirrhinum* flowers is composed of five petals and has dorsoventral asymmetry. The petals are colour coded as follows: ventral petal identity (yellow), lateral petal identity (red) and dorsal petal identity (blue). For simplicity, differences in the dorsal or ventral stamens are not considered. (Schwarz-Sommer, Davies, and Hudson, 2003).

## Using Antirrhinum as a Model Plant

The snapdragon, *Antirrhinum majus*, has been used as a model plant for the study plant genetics and development. Although *Arabidopsis thaliana* has been used as a model successfully to study plant architecture, *A. thaliana* does not provide conspicuous variation in phenotypes and can not extensively represent the existing species of flowering plants. *Antirrhinum* is a young genus and its species have a wide range of differences in morphology that can be crossed to each other to form fertile hybrids in cultivation. *A. majus* is one of about 20 *Antirrhinum* species that show a wide range of differences in morphology (Figure 1.8). Combined with classical and molecular genetics, this makes *A. majus* an ideal comparative angiosperm for the study of development and evolution (Schwarz-Sommer *et al.*, 2003).


**Figure 1.7 Aspects of *Antirrhinum majus* morphology.** Original book source: Prof. Dr. Otto Wilhelm Thomé *Flora von Deutschland, Österreich und der Schweiz* 1885, Gera, Germany

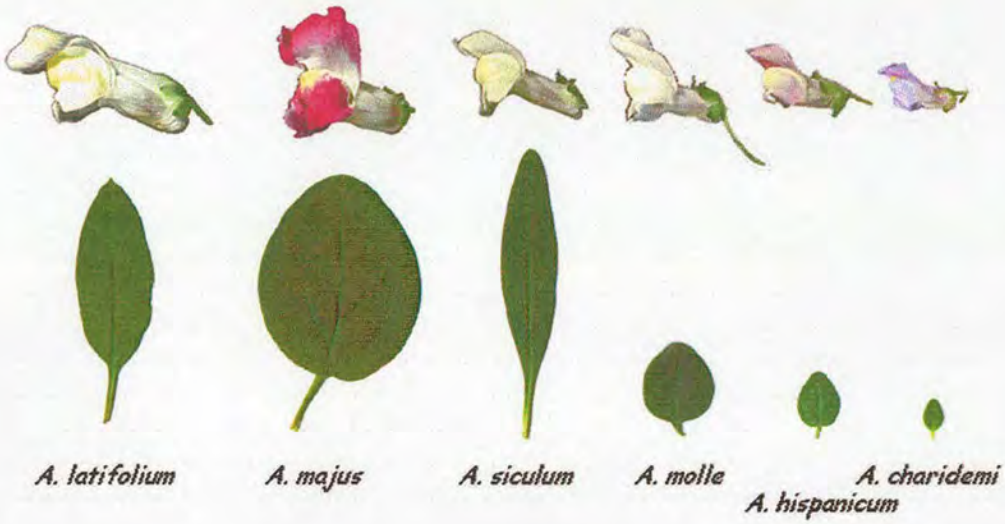



**Figure 1.8 Morphological differences between different Antirrhinum species.** Leaves and flowers of six of the 20 different Antirrhinum species are presented.

Mutant screens can provide opportunities to understand how genes control differences in organ size in both animals and plants (e.g. Stocker and Hafen, 2000; Mizukami and Fischer, 2000). This approach, however, can miss genes that are redundant or lethal when mutated, but which might differ in their activity between species. It is also not clear that the genes identified from mutations are likely to be the major contributors to evolutionary change.

Ideally the species used to identify genes underlying natural variation should have a wide range of variations and form fertile hybrids with each other. This is rare in the animal kingdom in which most hybrids between species are sterile. Species related to *Arabidopsis thaliana* would be an obvious choice but unfortunately close relatives differ in chromosome number and are unable to form fertile hybrids (Alonso-Blanco and Koornneef, 2000), this limits the range of morphological variation that can be investigated to that between ecotypes of *Arabidopsis thaliana*.

These limits do not apply to *Antirrhinum* species, making it an ideal model for the study of organ size and shape variation under natural evolution. Antirrhinum species have distinct morphologies and the organs (floral and

vegetative) vary considerably in size. All species can be crossed with each other and with cultivated *A. majus* to give fertile hybrids, allowing analysis of segregating hybrid populations. Unlike the situation with tomato fruit, organ size of cultivated *A. majus* does not differ greatly from that of wild *A. majus* relatives, suggesting that it has not experienced strong selection during domestication.

## Aim of the PhD

The primary aim of my PhD was therefore to understand the genetic basis for evolution of organ size in *Antirrhinum* by detecting quantitative trait loci underlying differences between two *Antirrhinum* species. I also examined the genetic basis for other traits in which two *Antirrhinum* species differed conspicuously, including plant size and branching, the density and morphology of trichomes and flower pigmentation.

# Chapter 2: MATERIALS AND METHODS

## 2.1 Plant Material

An F2 population (*n* = 107) was made from a single F1 plant that came from a cross between *Antirrhinum majus* (JI98) and *Antirrhinum molle*. The ovary parent, *Antirrhinum majus* had been obtained from the John Innes Centre (JIC), Norwich, United Kingdom and *A. molle* from the IPK, Gatersleben, Germany and was probably collected originally from Catalunia, Spain (Schwarz-Sommer *et al.* 2003). F1 seeds were obtained from Zsuzsanna Schwarz-Sommer, Max Planck Institute for Plant Breeding Research, Germany. Members of the F2 population were selected randomly at the seedling stage. F2 plants were grown individually in a 60 mm x 60 mm plug-system with Bulrush special *Antirrhinum* compost (Table 2.1), grown at 20~25°C/day, 16~20°C/night in the glasshouse in the Institute of Molecular Plant Science, University of Edinburgh.

**Table 2.1 The Bulrush Antirrhinum compost contents**

| MAIN INGREDIENT | |
|---|---|
| dark peat | 30% (by volume) |
| light peat | 60% |
| sod peat | 10% |

| ADDITIVES (per $m^3$) | | |
|---|---|---|
| base N:P:K 15:10:20 + trace elements | 0.75 | kg |
| Multicots 5 mini | 1.0 | kg |
| perlite-medium | 150.0 | l |
| lime/dolodust | 4.0 | kg |
| wetting agent | 0.4 | l |

All the F2 plants were also propagated vegetatively from cuttings for future DNA extraction. Although a linkage map of a F2 hybrid population (*n* = 92) of *A. majus* and *A. molle* had already been produced by Zsuzsanna Schwarz-Sommer and had been genotyped with RFLP and CAPS markers (Schwarz-Sommer *et al.* 2003), only three leaf traits had been scored. Unfortunately, the F1

parent of the original F2 mapping population had died. Therefore I produced a second F2 population ($n$ = 107) from a different F1 individual from the same *A. majus* and *A. molle* parents. In addition, F3 populations from both Zsuzsanna's and my F2 individuals were later grown together under the same conditions in the glasshouse to compare the phenotypic analysis results. A brief summary of how the plants are related are presented in Figure 2.1.

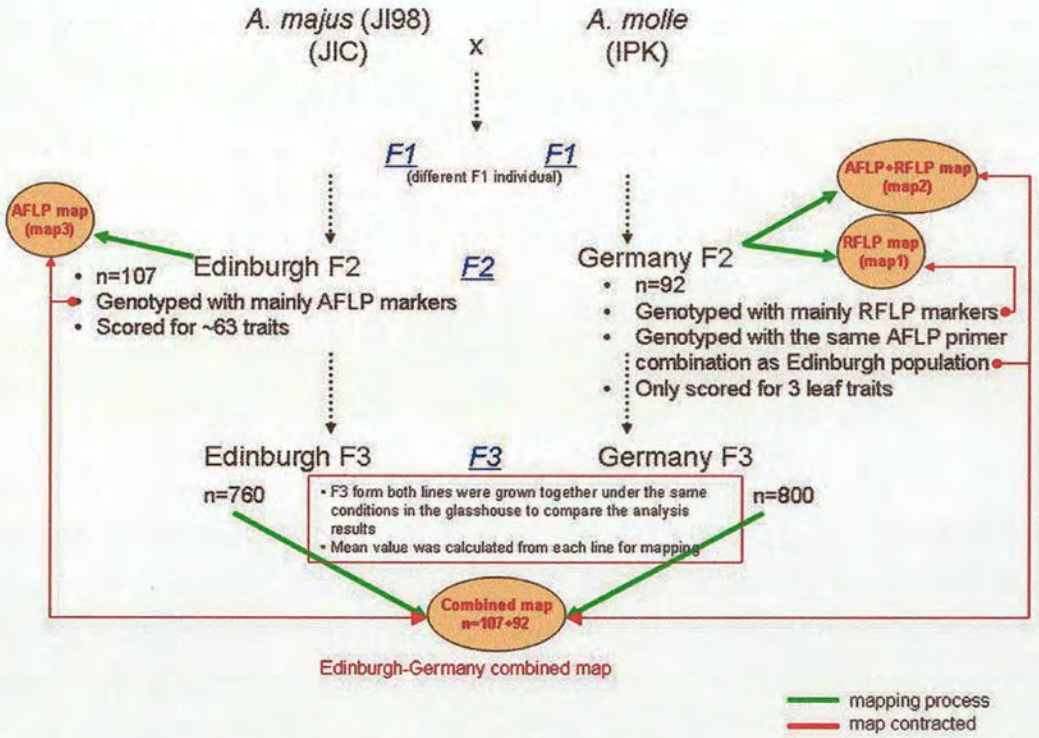



**Figure 2.1 Summary of how Edinburgh and Germany families were grown and correlated.** Both families were from the same parental cross, but from different F1 plants. Different genetic maps were constructed (shown in circular form). The QTL analysis was carried out from different population-map combinations (shown in green). The combined map used for F3 mapping was constructed from both F2 maps (map2 & 3).

## 2.2 Phenotypic Data Analysis

### F2 Population

Different vegetative and reproductive traits were scored in my F2 population ($n$ = 107). In total more than 50 phenotypic traits were scored for data analysis.

- **Leaf Measurements (Figure 2.2)**

Both leaves from the third internode above the cotyledons (cotyledons are at node 0) were harvested from each individual for digital imaging analysis. Firstly, leaves were stuck onto 2 mm x 2 mm graph paper and scanned as grey scale tiff image files. Secondly, images were loaded into ImageTool, with a built in function to calibrate grids on the graph paper as reference. Finally, a function was used to define leaf outlines as individual objects with a manual thresholding method. This allowed analysis of dimensional measurements (e.g. leaf area and leaf perimeter).

ImageTool is a free download analysis package (Donald Wilcox, Department of Dental Diagnostic Science, the University of Texas Health Science Center) (http://ddsdx.uthscsa.edu/dig/itdesc.html).

which offers a multi-function analysis including dimensional measurements (e.g. distance, perimeter and area). This gives an accurate analysis of organ size and shape (leaf, petal, epidermal cells *etc.*).

It was used to measure the following traits:

1. leaf area($mm^2$).
2. leaf perimeter(mm).
3. maximum leaf length(mm).
4. maximum leaf breadth(mm).

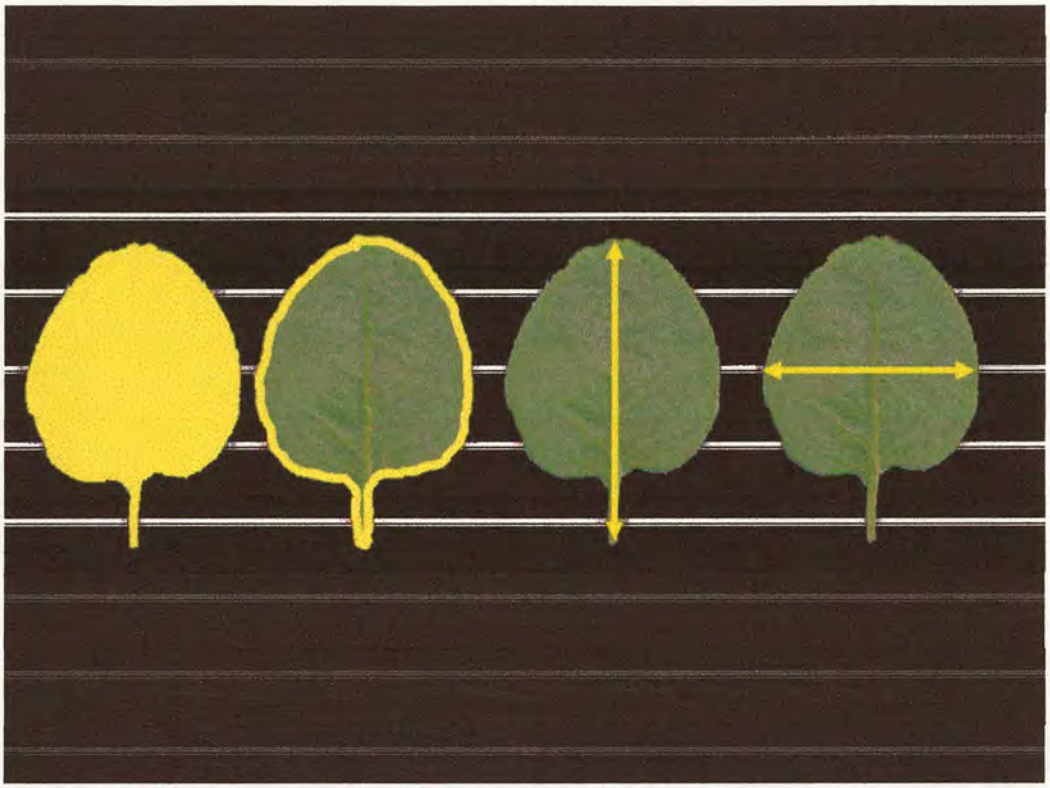


**Figure 2.2 Leaf traits measured.** The yellow highlights show the leaf traits measured with ImageTool; area, perimeter, maximum length and maximum breadth from left to right.

- **Flower Measurements (Figure 2.2.1)**

The first fully opened flower from each individual was collected and measured for 11 different traits with vernier callipers. Lobes from the right upper petal were cut from the flower and scanned and analysed as described for leaves. Anthocyanin was extracted from the upper petal for spectrophotometric analysis of pigment content. Fresh petal tissue was collected with a hole-puncher of 8 mm diameter, therefore the same area of tissue was harvested from each flower. Each sample was added to 0.5ml methanol-HCl solution (97% (v:v) methanol:3% hydrochloric acid) and left to extract overnight at 4°C. Finally the anthocyanin content was measured as absorption of light at 530 nm. Anthocyanin content is therefore shown as absorbance units per 50 $mm^2$ of petal tissue. Annabel Whibley from the John Innes Centre also scored red (anthocyanin) and yellow (aurone) pigment distribution in the middle lobe visually on an arbitrary scale of 1-5 for red pigment and 1-3

for yellow pigment. All these petal traits are given below.

1. upper petal image analysis.
   (1) Area($mm^2$).
   (2) Perimeter(mm).
   (3) Maximum length(mm).
   (4) Maximum breadth(mm).
2. upper petal anthocyanin analysis ($A_{530}$ units).
3. middle lobe anthocyanin estimate ($A_{530}$ units).
4. middle lobe yellow pigment estimate (arbitrary).
5. flower stalk (pedicel) length (mm)(F-1).
6. length of flower tube (mm)(F-2).
7. height of flower tube (mm)(F-3).
8. width between left and right mid-lobes (mm) (F-4).
9. length of gibba (mm)(F-5).
10. side length of upper petal (mm)(F-6).
11. width of lower lip (mm)(F-7).
12. the height between the point of two upper petals and lower lip (mm)(F-8).
13. length of style (mm)(F-9).
14. length of long anther filaments (mm)(F-10).
15. length of short anther filaments (mm)(F-11).


**Figure 2.2.1 Flower trait measurements.** The arrows show the traits measured (Scale bar = 1 cm).

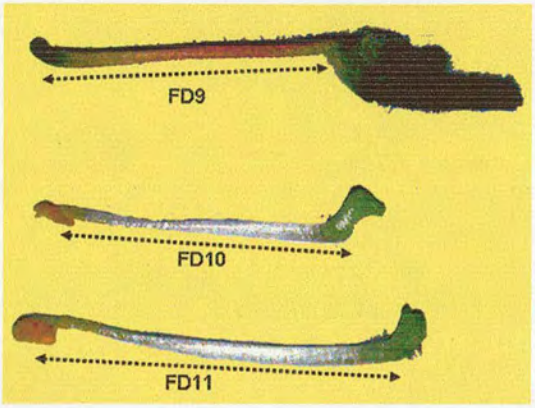



**Figure 2.2.1 (Continued).**

- **Other Growth Traits (Figure 2.2.2)**

Six other growth traits were scored. Flowering time was scored as days from sowing to full opening of the first flower. The total plant length was measured from the cotyledons to the first flowering node. The number of axillary branches was scored from the first node above the cotyledons to the first flowering node. The presence of at least one axillary branch from the cotyledons was also recorded. The internode diameter was measured at the midpoint of the three internodes above the cotyledons and averaged for the three internodes. These traits are presented below.

1. flowering time(day).
2. total plant length (cm).
3. node number.
4. number of branches and axillary buds.
5. presence of basal branch from axil of cotyledon.
6. internode diameter.

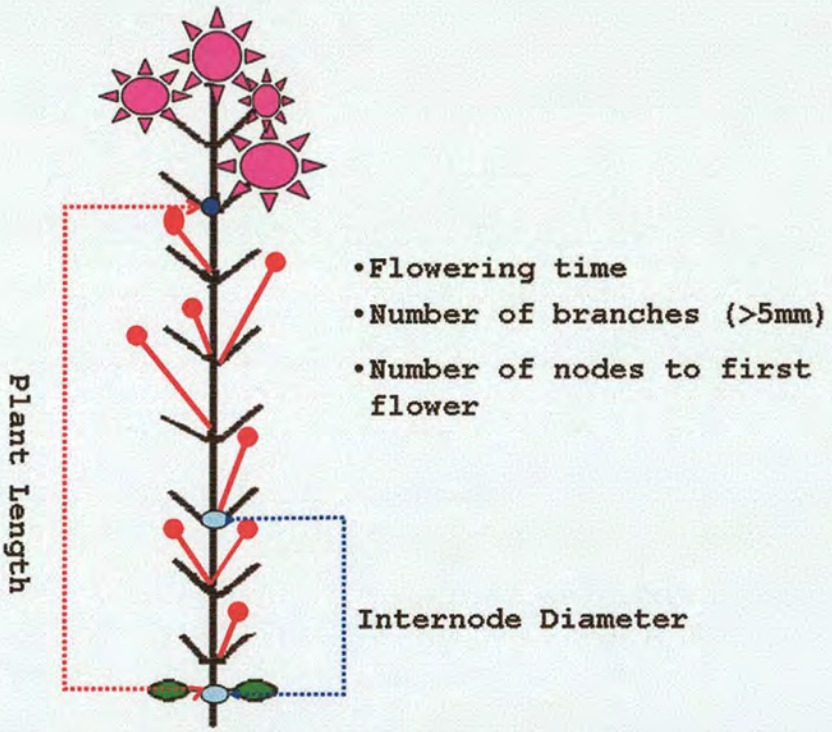



**Figure 2.2.2 Diagramatic explanation of how other growth traits were measured.** The plant length was measured from the cotyledons to the first flowering node. Internode diameter was measured as the mean of three base internodes. Axillary branches longer than 5 mm were counted. Flowering time was scored when the first flower was fully opened, and the node number was counted from the cotyledons to the first flower node.

- **Epidermis Structure**

Epidermis imprints were collected on microscope slides using Superglue. Leaf imprints were collected from the middle area of a leaf at node 4 from each individual and petal imprints from the middle area of a fully expanded upper petal lobe. The side of the tissue not being examined was stuck to Sellotape and the other side pressed onto a drop of Superglue on a slide. When the glue had hardened (usually after about 1 min) the tissue was peeled off using the Sellotape leaving an impression of the epidermal surface in the glue. If tissue remained attached to the glue, it was washed off with water and gentle scrubbing. Later on, imprints were photographed under a microscope at 200 X magnification. Images were saved as tiff files and printed, at the same scale, onto paper. Because the image analysis software was unable to distinguish individual cells, the outlines of about

twenty cells from each impression were traced onto acetate sheets and the tracings scanned. These were used for analysis with ImageTool, in the same way as described for whole leaf analysis. The mean cell roundness, elongation and compactness were also analysed, as presented below.

**Roundness:** Computed as:

$$(4 \times \pi \times area) \; / \; perimeter^2$$

The result gives a value between 0 and 1. The greater the value, the rounder the object. If the ratio is equal to 1, the object is a perfect circle, as the ratio decreases from 1, the object departs from a circular form. However, because the perimeter and area are not computed precisely, values above 1 may be obtained occasionally.

**Compactness:** Computed as:

$$sqrt(4 \times area \; / \; \pi) \; / \; major\ axis\ length$$

this provides a measure of the object's circularity that differs from roundness in using major axis length rather than perimeter. Basically the ratio of the feret diameter (the diameter of a circle having the same area as the object) to the object's length, will range between 0 and 1. At 1, the object is roughly circular. As the ratio decreases from 1, the object becomes less circular.

Roundness and compactness are similar to each other, except that roundness uses a object's perimeter and area while compactness is derived from an object's diameter to length ratio.

**Elongation:** The ratio of the length of the major (longest) axis to the length of the minor axis, orthogonal to the major axis.

A list of the traits that were scored for different part of epidermis is presented in Table 2.2.

**Table 2.2 List of the traits that have been scored for epidermis analysis.** Measurement units were in millimetres.

| | |
|---|---|
| **1** | abaxial leaf epidermis |
| | **1.1** area |
| | **1.2** perimeter |

**Table 2.2 (Cont.)**

| | |
|---|---|
| | **1.3** maximum length |
| | **1.4** maximum breadth |
| | **1.5** elongation |
| | **1.6** roundness |
| | **1.7** compactness |
| **2** | adaxial leaf epidermis |
| | **2.1** area |
| | **2.2** perimeter |
| | **2.3** maximum length |
| | **2.4** maximum breadth |
| | **2.5** elongation |
| | **2.6** roundness |
| | **2.7** compactness |
| **3** | abaxial upper petal epidermis |
| | **3.1** area |
| | **3.2** perimeter |
| | **3.3** maximum length |
| | **3.4** maximum breadth |
| | **3.5** elongation |
| | **3.6** roundness |
| | **3.7** compactness |
| **4** | adaxial upper petal epidermis |
| | **4.1** area |
| | **4.2** perimeter |
| | **4.3** maximum length |
| | **4.4** maximum breadth |
| | **4.5** elongation |
| | **4.6** roundness |
| | **4.7** compactness |

- **Trichomes (Figure 2.2.3)**

The density of long and short leaf trichomes (hairs) characteristic of *A. majus* and *A. molle*, respectively, were scored from both leaf and petal epidermis imprints. The value represents the number of each hair type present in one field of view of a dissecting microscope at 20x magnification (about 80 $mm^2$).

1. Abaxial leaf trichomes
2. Abaxial leaf long trichomes
3. Adaxial leaf trichomes
4. Adaxial leaf long trichomes
5. Abaxial petal trichomes

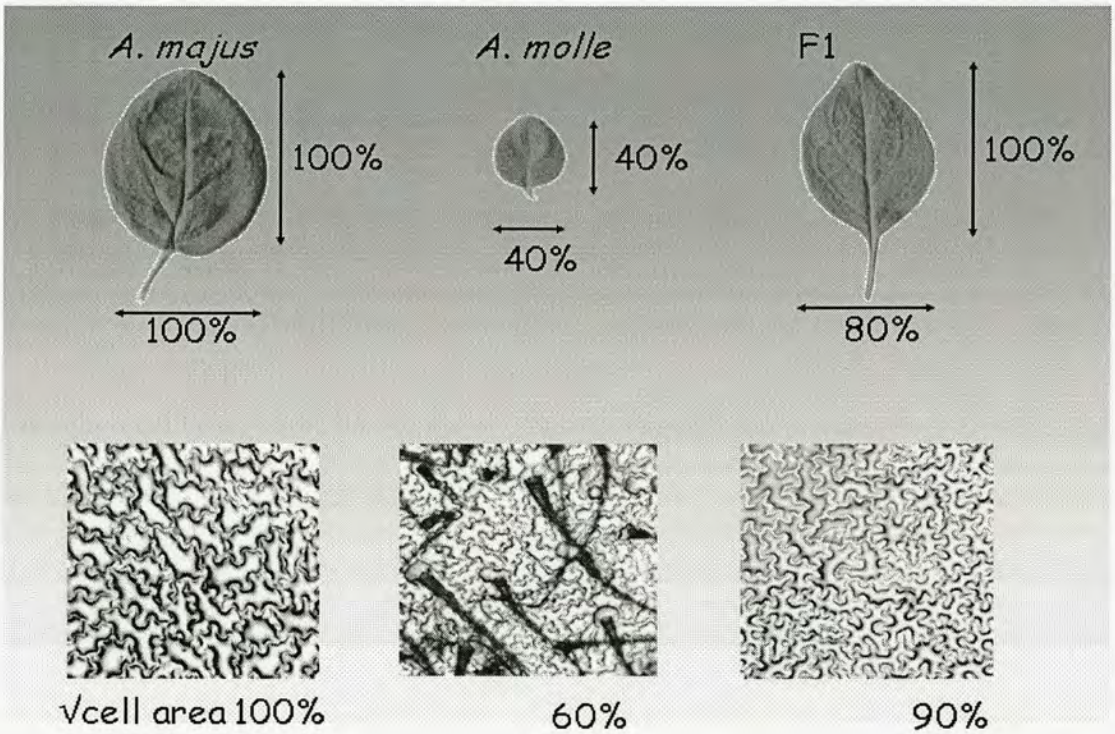



**Figure 2.2.3 The difference between *A. majus*, *A. molle* and F1 in leaf size, epidermal cells size and epidermal hair density.** The *A. molle* leaf has only about 40% of the length and breadth compared to *A. majus*. The F1 has the same length as *A. majus* but only 80% of the breadth. In cell area, *A. molle* has smaller cells and the F1 is intermediate between the two parents. The differences in cell size (converted to a linear scale by taking the square root of area) do not account for the total difference in leaf area, indicating that differences in cell numbers are also involved.

- **Correlation analysis**

All phenotype data were analysed to detect the correlation between each pair of traits using the correlation matrix function in the mathematical software MatLab®. The resulting correlation coefficients (*r*) have values from -1 to +1 where -1 means an absolute negative correlation, 0 means not correlated and +1 means an absolute positive correlation. Correlations were displayed graphically using the multidimensional scaling function of PAST (http://folk.uio.no/ohammer/past/), which clusters characters in 2D space so that their proximity best represents the degree of their correlation. If two traits are highly correlated, it suggests that they are controlled by the same genes or closely linked genes.

- **Leaf Shape analysis**

Principal Component Analysis (PCA) was used for analysis of coordinate leaf shape and size differences. PCA is a multivariate mathematical procedure to calculate a simplified visualization of data sets without losing experimental variances. Practically, correlated variances are transformed into a set of uncorrelated variances (principal components or PCs)

Each principal component is independent of the others. The first principal component is the combination of variances that explains most variation. The second principal component defines the next largest amount of variation and is independent to the first principal component. There can be as many possible principal components as there are variables (Paola and Francesca, 2002), but where there is covariance, the first few PCs can capture most of the total variance. The other PCs can be removed with minimum loss of information.

In this study, PCA was carried out using either the method described by Langlade *et al.* (2005) or a method that I developed myself.
A number of steps were involved in the Langlade method:

1. JPG formatted images of leaves were used.
2. Grids on the graph paper were used as a reference to scale leaf image to their actual size in Matlab.
   The outline of the leaf was reduced to a series of points (a 'points

model') in Matlab using the macro "pmcreate" provided by Nicolas Langlade. The points were either primary or secondary. Primary points were defined as the leaf tip, positions of maximum breadth, the junction between blade and petiole and the base of the petiole. These points were placed by hand. The secondary points were spaced equally between certain primary points in Matlab (Figure 2.2.4) and their positions adjusted by hand where necessary. The petioles were included in the analysis. Points models were rotated so that the midrib of each leaf was horizontal (i.e. the y co-ordinates of the leaf tip and petiole base were equal). The centroid (essentially the centre of mass) of each points model was calculated and the values in the points models translated so that centroids were set to the same position.

3. Corresponding points from different points models were identified using the macro "pmplace". For each point, the positions in the population of points models was saved in individual "_pm" files representing x and y coordinates of each point.
4. All the pm files were put together, using the macro "buildpdm" to calculate the mean position of each point, and therefore the mean leaf shape, the variance in the position of each point and the principal components capturing most of the variance in position of all the points.
5. Principal components explaining at least 99% of the total variance were calculated. The combination of PCs, mean positions of points and variances in position is referred to as a shape model. The effects of variation in each PC on the mean leaf shape were reconstructed graphically from this model.
6. The shape model was used to describe the shape and size of each leaf in the mapping population as standard deviations in each PC, from the displacement of each point in individual points models from the mean positions. Calculating the non-normalised values for each PC involved the function

$$[\,PC = pdm.P * (pts - pdm.Xm)\,]$$

Where **PC** is the component value corresponding to each individual, **pdm.P** is the surviving eigenvectors, **pts** is the coordinate axis of each point and the **pdm.Xm** is the data of mean shape (mean value).

Normalized values were calculated in standard deviation (dev) units as:

$$[\text{dev} = \text{PC}/\text{sqrt}(\text{pdm.b})]$$

where **dev** is the standard deviation value corresponding to each individual, **PC** is the PC value from each individual, and the **pdm.b** is the corresponding eigenvalues.

7. The quantitative descriptions of leaf shape and size as PC values was used in QTL analysis to detect the genes underlying each PC.

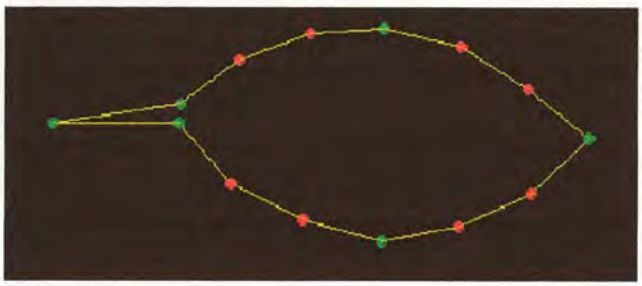


**Figure 2.2.4 Points used to capture leaf shape.** Primary points (green circles) are placed at key landmarks and secondary points (red circles) are automatically spaced at equal intervals between primary points.

My method differed from that of Nicolas Langlade's in a number of ways.

1. For better quality of leaf outlines, tiff formatted images of leaves were used.
2. Images were scaled to actual size, as before, but rotated manually so that the midrib was vertical.
3. ImageTool was used to select leaf outlines and the outlines represented as a larger number of points. The spacing of points was the default set by ImageTool, but was equal and the same for all images. The smallest leaves were represented by at least 100 points. The positions of the points were saved as *.conts files.
4. A macro was written for ImageTool to define the leaf tip (A), the petiole-blade junctions (B,C), and the proximal end of the petiole (D; Figure 2.2.5). The Cartesian co-ordinates of these points were saved as a "*.points" file. One advantage of this method was that simple measurements (e.g. length or width) could be extracted from points files.
5. For each leaf, the outlines (*.conts file) was combined with its *.points files in Matlab. Matlab was used to remove the petiole, by

eliminating points with y coordinate values less than those of points B or C, and to calculate the middle point of the petiole-blade junction (E).

6. Leaf outlines were reduced to ten points. Here I used $\gamma$ and $\theta$ coordinates ($\gamma$ is the distance from point E to the leaf outline along a line at angle $\theta$ to the horizontal). Fixed increments of $\theta$ were used to identify 14 points on the outline, including points A ($\theta = 90^{\circ}$) and B or C ($\theta = 0$; Figure 2.2.5). The coordinates of the reduced set of points were saved into *_pm" files. In this study, leaves were analysed as left and right parts separately, demarcated by the primary vein (AE). This should give a more specific analysis as sometimes leaf growth form is not always symmetrical (Figure. 2.2.5).
7. Points were translated so that point E had the same coordinates in each image. Points from the same angles in each individual's *_pm file were put together for principal component analysis.
8. PCs accounting for at least 95% of the total variance were calculated and shown as shape model changes (Figure 2.2.6).

One disadvantage of this method was that it could not easily describe the shape of individual leaves as values for the PCs and therefore QTL responsible for variation could not be identified. Therefore the Langlade method was used for all subsequent analysis. Two shape models were used to describe the variance in leaf shape and size in the *A. majus* x *A. molle* F2 population. The first was built from the data from leaves of this population. The second had been built from leaves of an F2 population of *A. majus* x *A. charidemi* by Nicholas Langlade. Use of the *A. majus* x *A. charidemi* model allowed direct comparisons between the two populations.

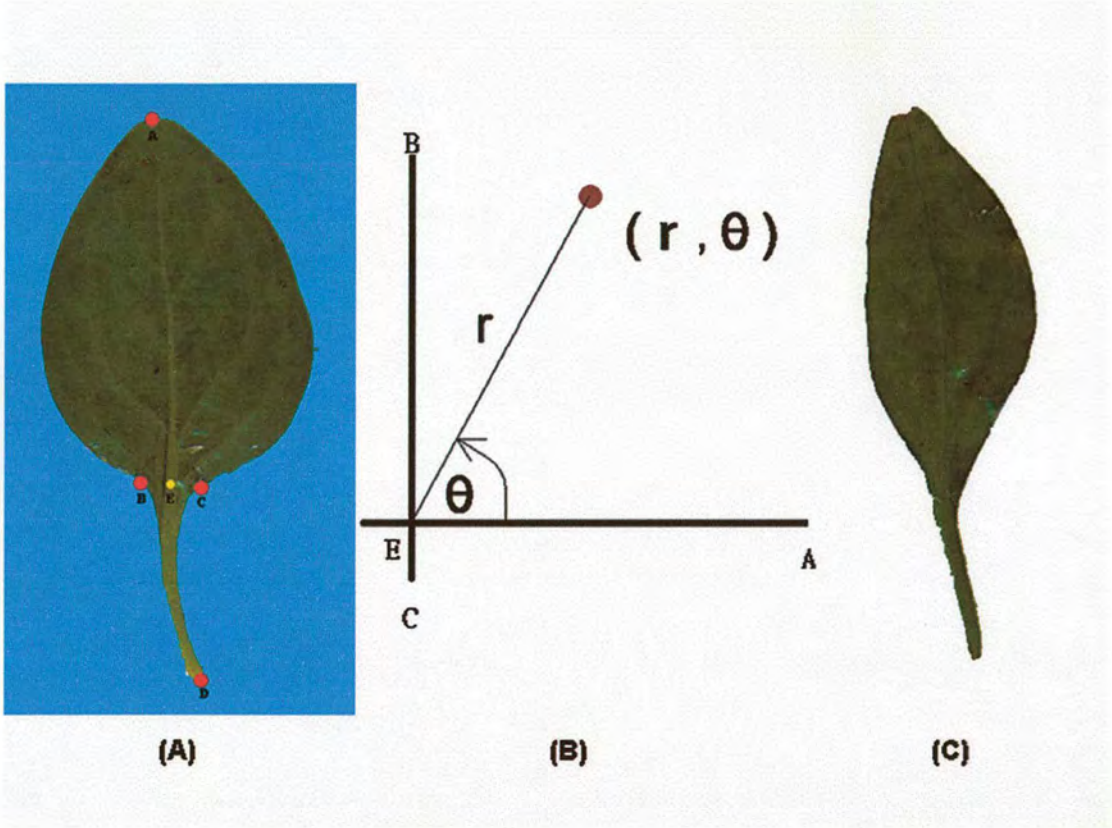



**Figure 2.2.5** **(A)** The points defined for the major leaf outline. Leaf tip (A), left petiole-blade junction (B), right petiole-blade junction C), base of petiole (D) and the middle of the BC line(E). **(B)**: How points on the leaf outline were defined. r is the distance from point E to leaf outline, θ is the angle from a line perpendicular to the midvein (AE). **(C)**: The picture shows that growth of some leaves was not symmetrical.

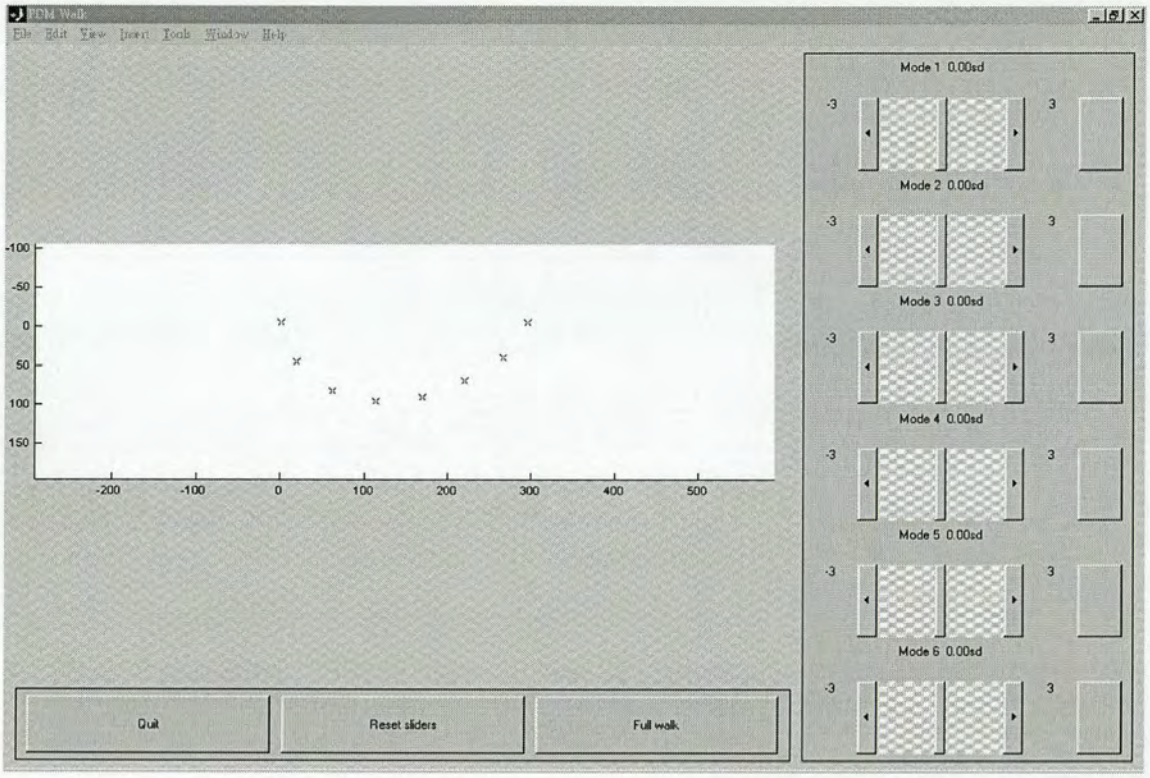



**Figure 2.2.6 MatLab workspace showing how leaf shape and size changes with variation in different principle components.** Only half the leaf points are shown in this figure.

## F3 population

To compare the morphological differences and QTL analysis between the Edinburgh and German F2 mapping populations, an F3 population was grown from both populations (107 Edinburgh F2 lines, and 96 German lines) under the same conditions. More than eight individuals from each line were grown individually in a 60 mm x 60 mm PlugIt system with Bulrush's *Antirrhinum* compost, at 20~25°C/day, 16~20°C/night temperature in the glasshouse in the Institute of Molecular Plant Science, University of Edinburgh. F3 seeds of the German line were provided by Schwarz-Sommer Z., Max Planck Institute for Plant Breeding Research, Germany.

- **Leaf Analysis**

Leaves were collected from the same node as for the F2 analysis, placed on 2 mm x 2 mm graph paper and scanned as digital image files. An example of two image files is shown in Figure 2.2.7. Later, images were calibrated using the grids on the graph paper as reference and analysed with ImageTool. An internal function was used to define leaf outlines as individual objects with manual thresholding methods. This allowed analysis of dimensional measurements (e.g. leaf area, leaf perimeter etc). The traits were as follows

1. leaf area($mm^2$).
2. leaf perimeter(mm).
3. maximum leaf length(mm).
4. maximum leaf breadth(mm).

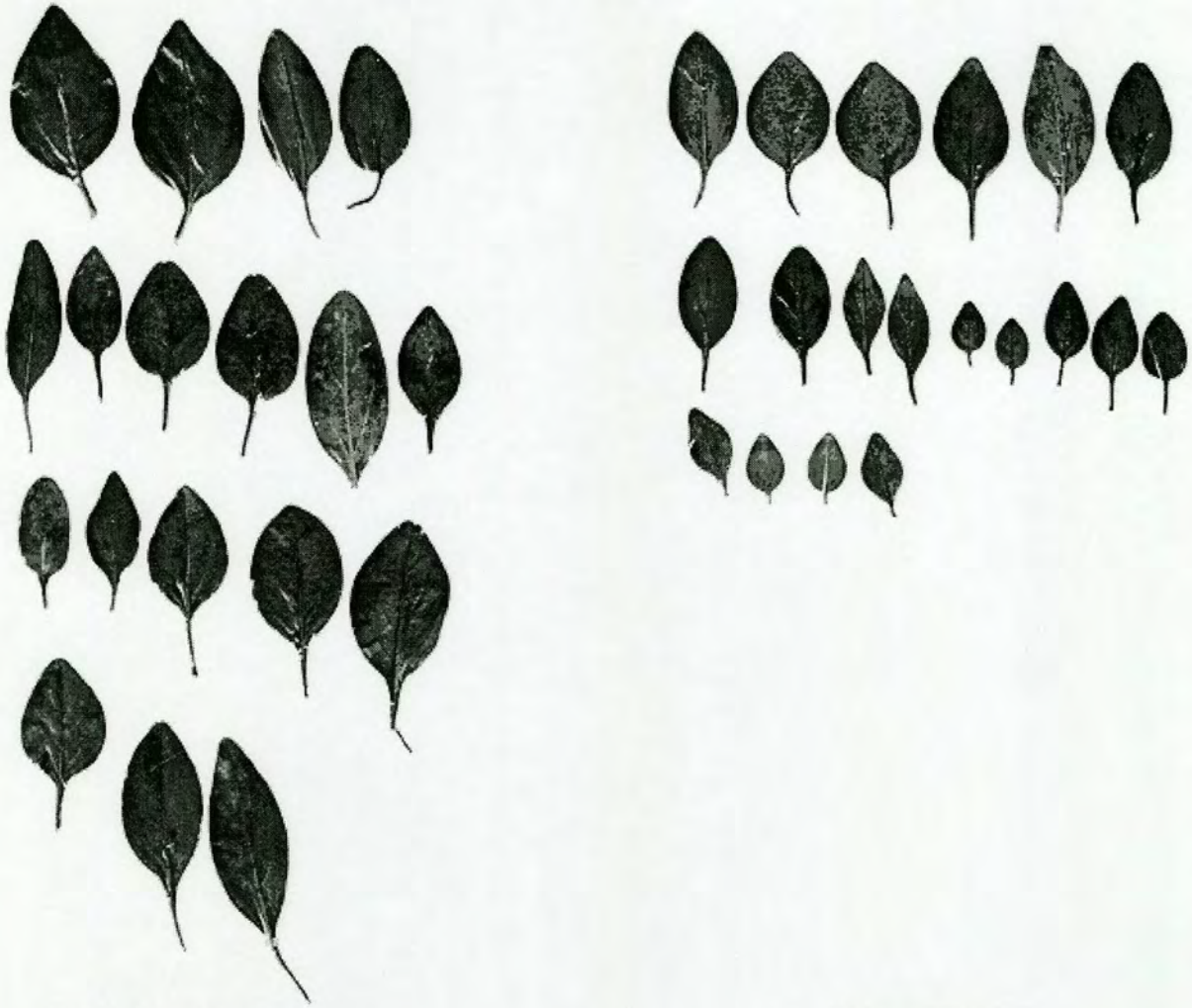



**Figure 2.2.7 Leaves from F3 lines showing different segregation.** Each image represents leaves from members of the same F3 family (i.e. derived from the same F2 parent) and both node 3 leaves from each F3 plant was used where possible. Leaves from the same plant are adjacent.

## 2.3 Genotyping Procedures

### Molecular Markers

- **Amplified Fragment Length Polymorphism (AFLP)**

Amplified fragment length polymorphism (AFLP) analysis is a polymerase chain reaction (PCR)-based marker system which gives a quick way to screen genetic diversity (Vos *et al.*, 1995). It uses the advantages of PCR to generate hundreds of highly replicable markers from DNA fragments and thus high-resolution genotyping. This makes AFLP superior or equal to markers like random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), or microsatellites, except that AFLP methods primarily generate dominant rather than co-dominant markers.

AFLP involves three steps:

**(i)** Restriction of DNA and ligation of oligonucleotide adapters (Figure 2.3). One restriction enzyme - in this case *Pst* I, recognises a 6 bp sequence and cuts infrequently and the second enzyme - here *Mse* I - recognises a 4 bp sequence and cuts more frequently. Therefore almost all fragments with a *Pst* I adaptor at one end will have an *Mse* I adaptor at the other.

**(ii)** Pre-amplification with primers corresponding to the adaptor sequences followed by selective amplification of sets of restriction fragments (Figure 2.3).

**(iii)** Separation and analysis of the amplified fragments.

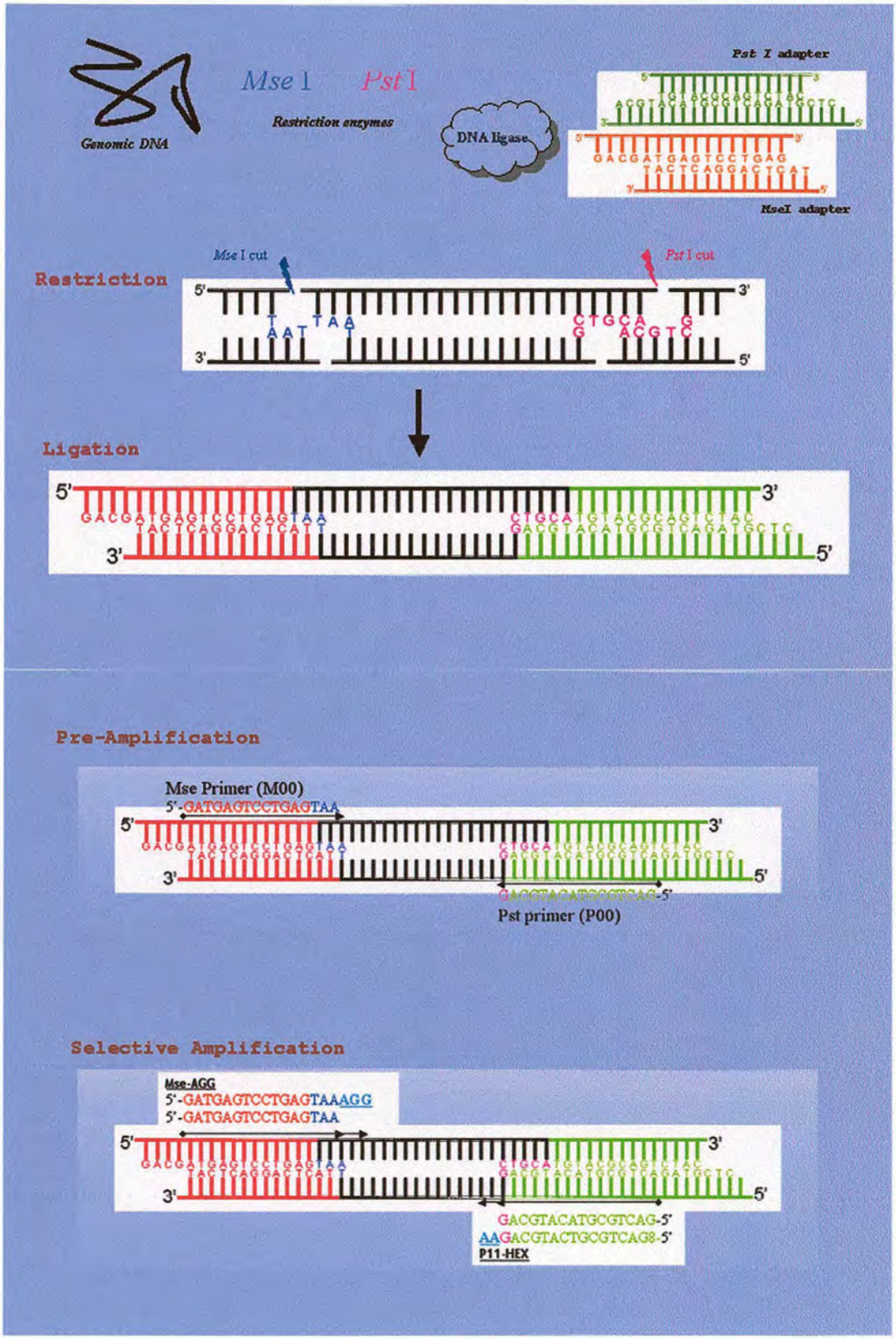



**Figure 2.3 AFLP procedures.** Restriction enzymes, *MseI* and *PstI* were used. Three major relations were involved: **Restriction:** genomic DNA was cut by restriction enzymes. **Ligation:** *MseI* and *PstI* adapters were linked to specific DNA fragments. **Pre-Amplification** and **Selective Amplification:** amplification of DNA fragments with specific nucleotides 3′ of the restriction sites.

In this study, we produced AFLP fragments by digestion with *Pst* I and *Mse* I enzymes. In the selective amplification, the *Pst* I + 3 primers were labelled with fluorescent dyes (6-FAM, HEX, and TET) that could be detected by an ABI 310 sequencing machine.

- **Restriction of DNA and Ligation of Oligonucleotide Adapters**

Genomic DNA (2.5 µg) in a total volume of 20 µl was digested with two restriction enzymes, *Pst* I and *Mse* I, at 37°C for 2 hours. Annealed oligonucleotide adapters were ligated to 20 µl of the digested DNA, by incubation at 37°C for 3 hours then the reactions stored at -20°C.

Details of restriction and ligation protocol are show below

**5x RL buffer:**

| | |
|---|---|
| One-For-All-buffer (Promega) | 500 µl |
| DTT 0.1 M | 250 µl |
| BSA 10 µg 7µl | 25 µl |
| Distilled $H_2O$ | 225 µl |

**Restriction reaction components**

| | |
|---|---|
| DNA (250ng/µl) | 10.00 µl |
| *Pst* I 20U/µl | 0.25 µl |
| *Mse* I 10 U/µl | 0.50 µl |
| 5x RL buffer | 4.00 µl |
| $dH_2O$ | 5.25 µl |
| TOTAL VOL. | 20.00 µl |

Adaptors (50 µM each oligonuleotide in 1x RL buffer) were annealed by heating to 60°C followed by slow cooling to room temperature.

**Ligation reaction components**

| | |
|---|---|
| 5x RL buffer | 1.000 µl |
| *Pst* adapter 50 pmol/µl | 0.500 µl |
| *Mse* adapter 50 pmol/µl | 0.500 µl |
| rATP 10 mM | 0.500 µl |
| T4 ligase 5 U/µl | 0.125 µl |
| $dH_2O$ | 2.375 µl |
| TOTAL | 5.000 µl |

***Pst* I adaptor sequences**

5′-TGTACGCAGTCTAC-3′
3′-ACGTACATGCGTCAGATGCTC-5′

***Mse* I adaptor sequences**

5′-GACGATGAGTCCTGAG-3′
3′-TACTCAGGACTCAT-5′

## • Pre-amplification and Selective Amplification of sets of Restriction Fragments

Pre-amplification involved *Pst* I primer P00 (see below) and *Mse* I primer (M00) together. Digested and ligated DNA (5.0 µl) was added to 15 µl of pre-amplification mixture and PCR run for 35 cycles, using the condition shown below. An aliquot of 5µl of the pre-amplification reaction was run on a 1% agarose gel to confirm amplification.

Pre-amplification primer sequences

**P00**

5′ -GACTGCGTACATG**CAG**-3′

**M00**

5′ -GATGAGTCCTGAG**TAA**-3′

**Pre-amplification reaction components**

| | |
|---|---|
| Digested and ligated DNA | 5.0 µl |
| 10x PCR buffer | 2.0 µl |
| 25 mM $MgCl_2$ | 2.4 µl |
| dNTP 10 mM | 0.4 µl |
| *Pst* primer (P00) 10 µM | 0.6 µl |
| *Mse* primer (M00) 10 µM | 0.6 µl |
| Taq 1 U/µl | 0.2 µl |
| $dH_2O$ | 8.8 µl |
| TOTAL | 20.0 µl |

**Pre-Amplification cycling conditions**

| | | |
|---|---|---|
| 72°C | 2 min | |
| 94°C | 20 s | |
| 56°C | 30 s | |
| 72°C | 2 min | } 35 times |
| 60°C | 30 min | |

Selective amplification reaction involved the extension of fragments with more specific *Pst* and *Mse* primers (Table 2.3). In this study, six different *Pst* and *Mse* primer combinations were used for amplified DNA fragments. The *Pst* primers were labelled with different fluorescent dyes at their 5′ ends (see below). Diluted pre-amplification (2.0 µl of a 1:5 dilution in TE) was used as a template in a 10 µl PCR. PCR cycling condition are shown below

*Mse* I primer combination used for AFLP analysis

1) P11-M00 + AGG
2) P11-M00 + AGA
3) P11-M00 + CAC
4) P12-M00 + AGC
5) P12-M00 + ACA
6) P14-M00 + AGC

**Table 2.3 Selective amplification primer sequences**

| | |
|---|---|
| **P11-HEX(yellow)** | **$M_{AGG}$** |
| 5′-8GACTGCGTACATGCAGAA | 5′-GATGAGTCCTGAGTAA**AGG** |
| **P11-TET(green)** | **$M_{AGA}$** |
| 5′-TET-GACTGCGTACATGCAGAA | 5′-GATGAGTCCTGAGTAA**AGA** |
| **P11-6FAM(blue)** | **$M_{CAC}$** |
| 5′-6GACTGCGTACATGCAGAA | 5′-GATGAGTCCTGAGTAA**CAC** |
| **P12-6FAM(blue)** | **$M_{AGC}$** |
| 5′-6GACTGCGTACATGCAGAC | 5′-GATGAGTCCTGAGTAA**AGC** |
| **P12-HEX(yellow)** | **$M_{ACA}$** |
| 5′-8GACTGCGTACATGCAGAC | 5′-GATGAGTCCTGAGTAA**ACA** |
| **P14-TET(green)** | |
| 5′-9GACTGCGTACATGCAGAT | |
| **P14-6FAM(blue)** | |
| 5′-6GACTGCGTACATGCAGAT | |

*Selective nucleotides are shown in red.

**Selective amplification reaction components**

| | |
|---|---|
| Diluted pre-amp | 2.0 µl |
| 10x PCR buffer | 1.0 µl |
| 25 mM $MgCl_2$ | 0.6 µl |
| dNTP 2 mM | 1.0 µl |
| Pst +3 primer 1 mM | 0.5 µl |
| Mse + 3 primer 5 mM | 0.5 µl |
| Taq 1U/µl | 0.05 µl |
| $dH_2O$ | 4.35 µl |
| TOTAL | 10.0 µl |

**Selective Amplification cycling conditions**

| | |
|---|---|
| 94°C | 2 min |
| 94°C | 20 s |
| 66°C | 30 s |
| 72°C | 2 min |
| 94°C | 20 s |
| 65°C | 30 s |
| 72°C | 2 min |
| 94°C | 20 s |
| 64°C | 30 s |
| 72°C | 2 min |
| 94°C | 20 s |
| 63°C | 30 s |
| 72°C | 2 min |
| 94°C | 20 s |
| 62°C | 30 s |
| 72°C | 2 min |
| 94°C | 20 s |
| 61°C | 30 s |

| | |
|---|---|
| 72°C | 2 min |
| 94°C | 20 s |
| 60°C | 30 s |
| 72°C | 2 min |
| 94°C | 20 s |
| 59°C | 30 s |
| 72°C | 2 min |
| 94°C | 20 s |
| 58°C | 30 s |
| 72°C | 2 min |
| 94°C | 20 s |
| 57°C | 30 s |
| 72°C | 2 min |
| 94°C | 20 s |
| 56°C | 30 s |
| 72°C | 2 min |
| 60°C | 30 min |
| 4°C | |

} 20 times

## • Gel Analysis of the Amplified Fragments

Products carrying the fluorescent dyes (6-FAM, HEX and TET) could be detected by the ABI 310 GeneScan sequencing machine. For detection, 2 µl of final selective amplification products from each of three different reactions (each involving a different dye) were combined with 15.5 µl of a master mix of HiDi formamide mix (ABI) plus TAMRA 500 size standard (ABI), heated to denature DNA and run in the GeneScan machine by Anna Montazan (School of Biology Squencing Service, Edinburgh University). Running condition are shown below

- Filter set : C
- Injection time : 15 s
- Injection kV : 15 kV
- Run kV : 13 kV
- Run time : 35 mins
- Vol of sample : 6 µl
- Size standard : Tamra 500

Tamra 500 size standard set consists of a mix of single-stranded fragments, labelled with TAMRA, with the following sizes (in bp) 500, 490,450,400, 350, 340, 300, 200, 160, 150, 139, 100, 75, 50 and 35.

The output trace files from GeneScan were processed with GeneScan software which calibrates them against the size standard.

## Genographer

The output data from GeneScan were analysed with Genographer (http://hordeum.oscs.montana.edu/genographer/), which creates virtual gel images from ABI trace files and allows automated scoring of AFLP bands. AFLPs that were polymorphic between the two parents were analysed as dominant or co-dominant markers (Figure 2.3.1).

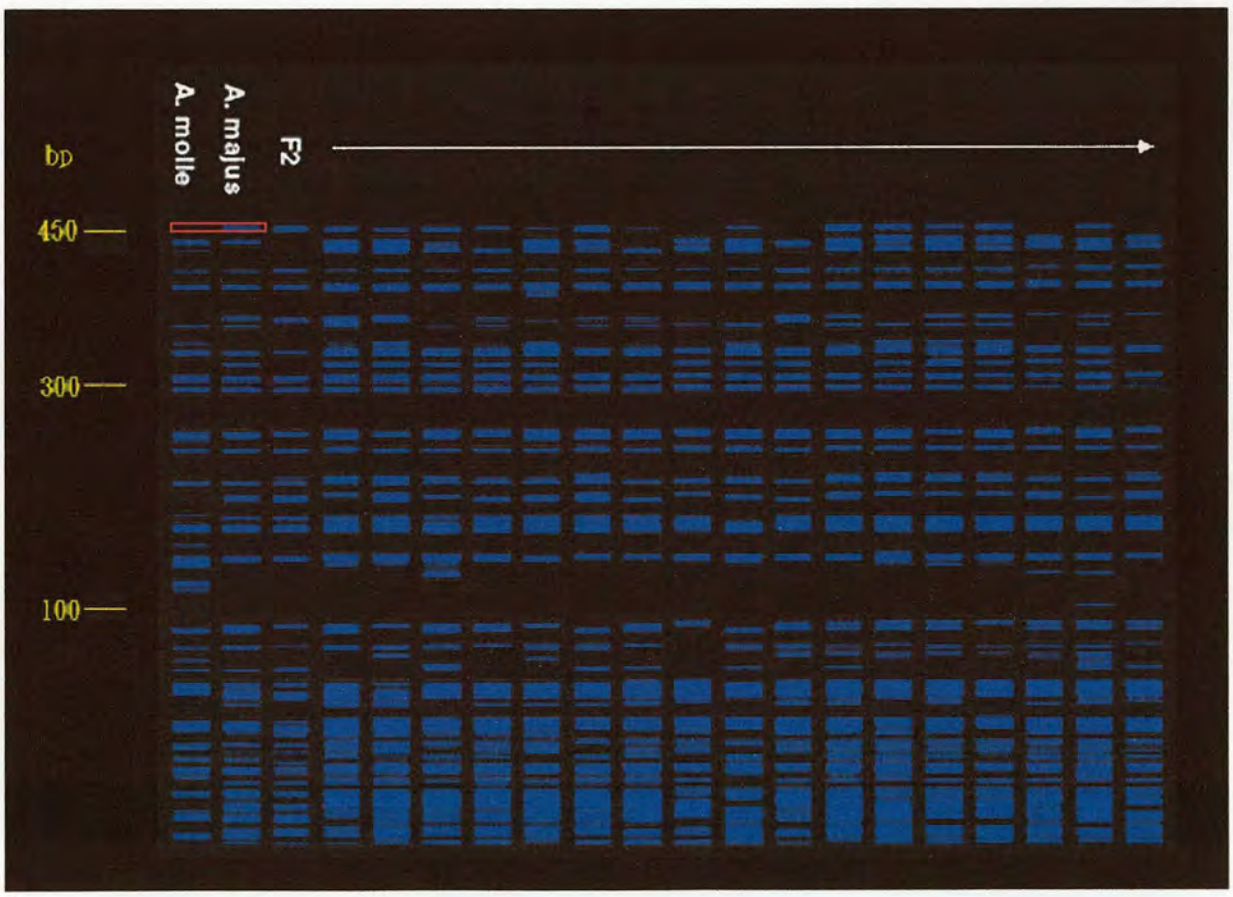



**Figure 2.3.1 Part of a virtual gel visualised with Genographer.** One AFLP that is polymorphic in the two parents (*A. molle* and *A. majus*) is highlighted. This shows segregation in the F2 progeny.

Automated scoring of segregating bands involved three steps:

1. The signal in the different samples was normalised. A bin was created around each segregating AFLP by clicking the mouse on the band. The size range of the gel could be changed to show bands that were hard to distinguish if two or more bands were close to each other (Figure 2.3.2).
2. The bands within each bin were visualised as thumbnails showing the fluorescence intensity of each band as a peak. For scoring of dominant AFLP markers, a threshold fluorescence value, above which the band was scored as present, was set. For co-dominant scoring, three different thresholds were set (see below; Figure 2.3.3).
3. The scoring of the marker was carried out by clicking the "Analysis" button on the left of the screen. The output file could be saved as a text file by selecting the "FileExport" function.

In some conditions (e.g. when samples were not properly aligned), bands were scored manually.

The following scoring system was used for genotypes. For dominant markers the presence of an AFLP from the *A. majus* parent was scored as D and its absence (i.e. a plant homozygous for the *A. molle* allele) as B. The presence of an AFLP from the *A. molle* parent was scored as C and its absence (i.e. an *A. majus* homozygote as A). For co-dominant scoring, plants clearly homozygous for either the *A. majus* or *A. molle* AFLP were scored as A or B, respectively and heterozygotes as H. When the height of the peak was intermediate between those expected for a homozygote and a heterozygote, the genotype was scored as either C or D, representing the presence of an *A. molle* or *A. majus* band, respectively.


**Figure 2.3.2 Creation of a bin for scoring a segregating AFLP band.**

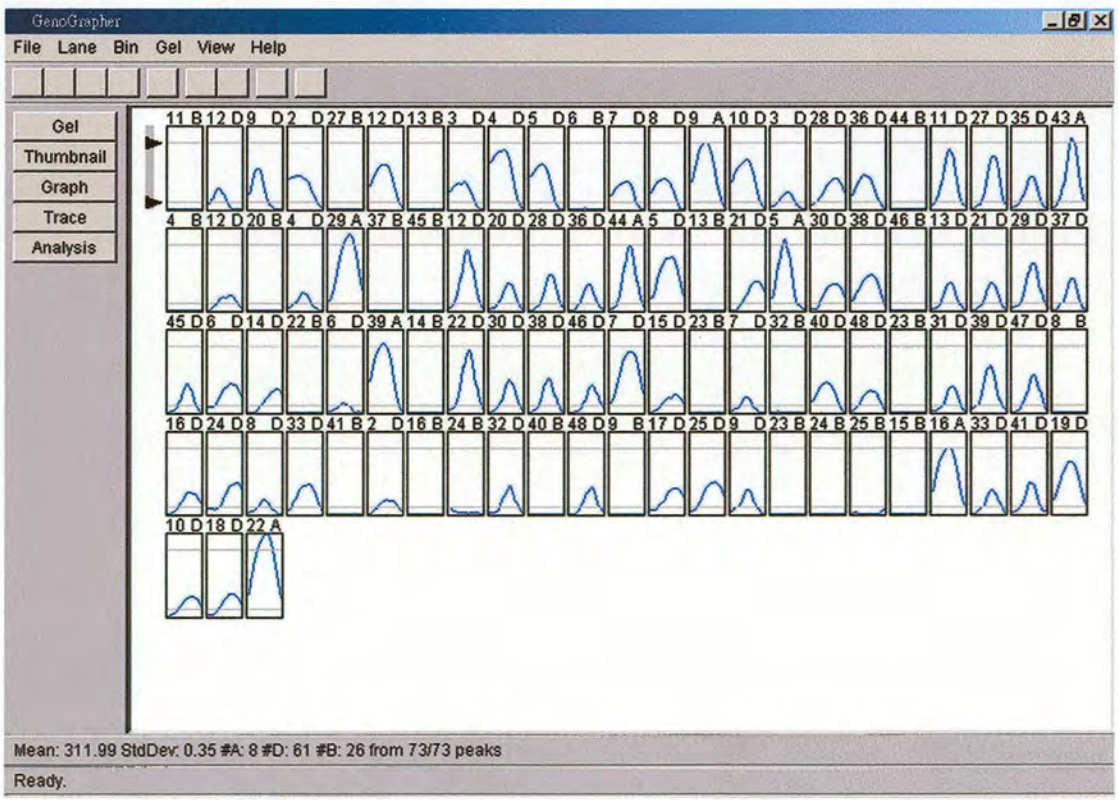



**Figure 2.3.3 Fluorescence within the binned region of each trace represented as thumbnails The thresholds for scoring as a dominant AFLP marker from *A. majus* are shown.**

## JoinMap®

Molecular linkage maps were calculated with JoinMap® 3.0 (Van Ooijen and Voorrips, 2001) (http://www.kyazma.nl). We used 164 amplified fragment length polymorphism (AFLP) fragments scored as dominant markers and twenty eight co-dominant cleaved amplified polymorphic sequences (CAPS) markers. Most CAPS loci were genotyped by Amanda Borking. The loci with co-dominant CAPS alleles were scored as homozygous *A. molle* (b) homozygous *A. majus* (a) or heterozygote (h). Kosambi's function were used calculate the map (Figure 2.3.4). Most linkage groups were resolved with a LOD (log of odds) score of >3.

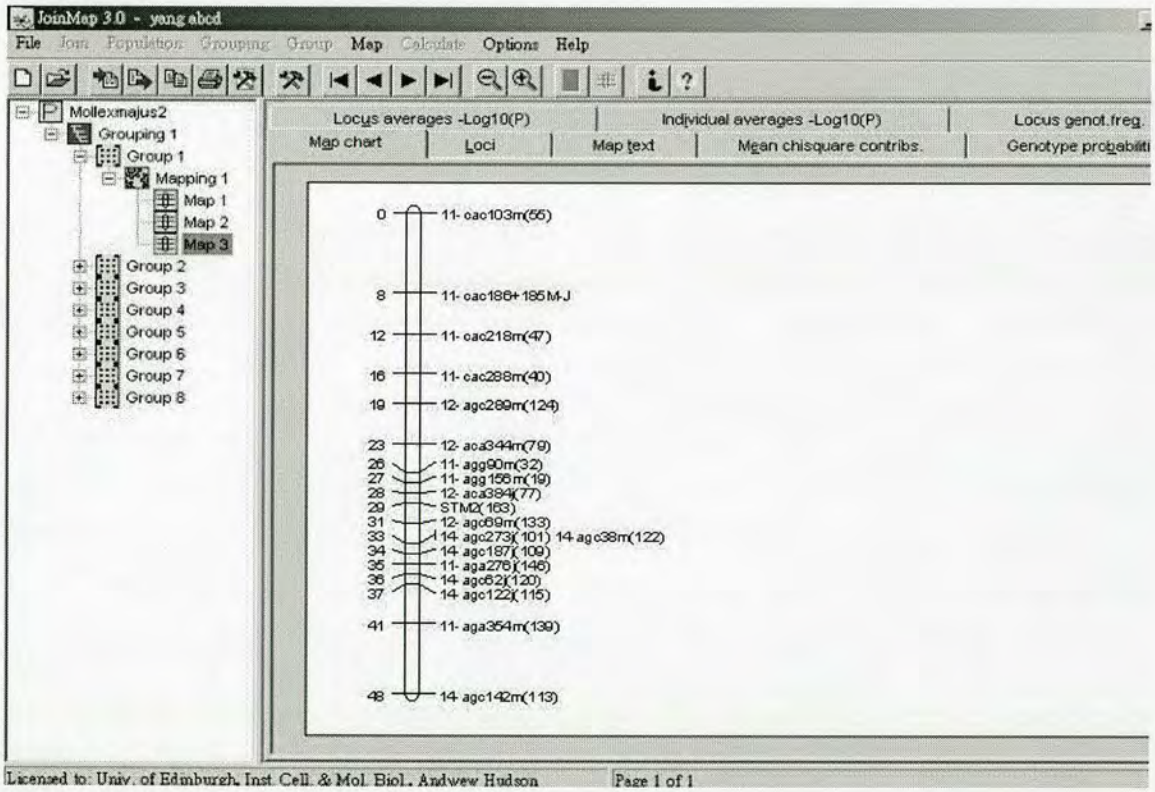



**Figure 2.3.4 Linkage map generated in Joinmap with locus names and map positions.**

## Combined Map of Edinburgh and Germany Population

The first *A. majus* x *A. molle* linkage map was built mainly with restriction fragment length polymorphism (RFLP) markers (Schwarz-Sommer *et al.*, 2003); however the linkage maps in my population were built mainly with amplified fragment length polymorphisms (AFLP). Because the RFLP map was not fully comparable with AFLP maps, a new AFLP linkage map of Schwarz-Sommer's F2 population was built using the same primer combinations as my maps (F2 DNA samples were kindly provided by Z. Schwarz-Sommer). Later, both populations were joined together as one population (n = 203) to build maps with common markers. Both F3 populations were analysed with the combined map for QTLs (Figure. 2.3.5).

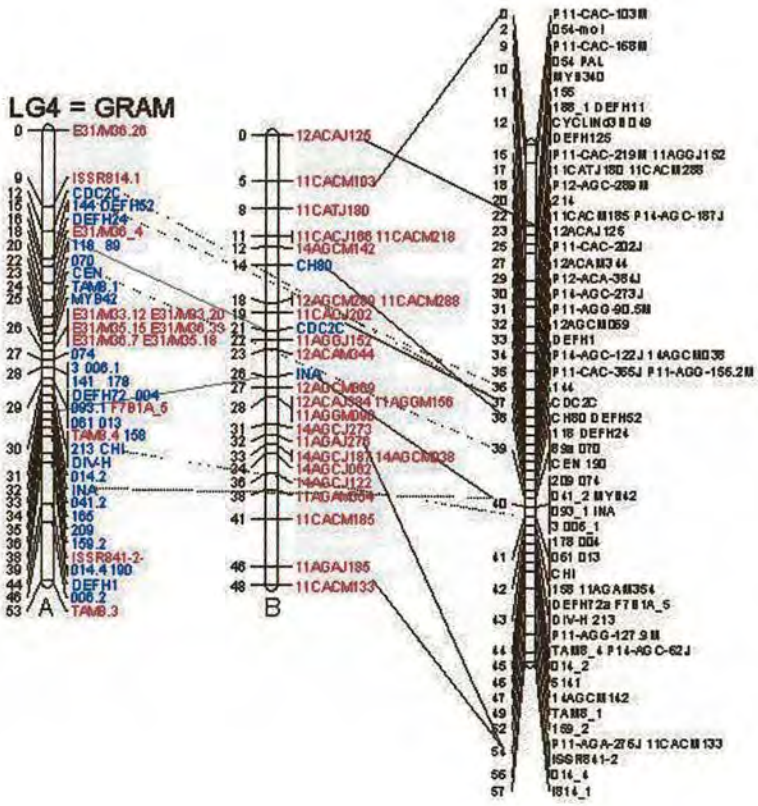



**Figure 2.3.5** The maps show the alignment between the first population (left), my population (middle) and the combined map at the right. Markers mapped in more than one population are joined by lines.

## 2.4 Quantitative Trait Loci Mapping

### Association of Molecular Markers with Qualitative Traits

A genetic map with 192 molecular markers (166 AFLP markers and 26 CAPS markers) was built for my F2 population by Amanda Borking and used for QTL analysis of my F2 population. This map is described in detail in Results.

### Quantitative Trait Locus Analysis

A statistical analysis for Quantitative trait loci (QTL) was carried on with a web based mapping programme called QTL-express (http://latte.cap.ed.ac.uk/)(Knott *et* al., 2002) provided by the Institute of Evolutionary Biology, The University of Edinburgh.

### F2 Data Formats

Data entry for QTL-express analysis requires three separate files which contain the following information:

1. Genotype data (i.e. the genotype for each individual at each marker locus).
2. Map information (i.e. marker positions on the linkage groups).
3. Phenotype data (i.e. traits). All the entry data files are entered in plain text (*.txt) with space or tab separated data. An example is shown below

### Genotype data

This example shows the format of genotype data:

```
192
11CACM133   11AGAJ185   11CACM185   11AGAM354   14AGCJ122
JI98  Molle F1    F2
M     F
0
1     F1_m  F1_f  M     F2    1     1     2     2     2     2
      1     1     1     1
2     F1_m  F1_f  M     F2    0     0     0     0     0     0
      0     0     1     2
```

**Line 1**: Number of markers

**Line 2**: List of names of the markers

**Line 3**: Names given to each of the generations. For the F2 cross there must be two grandparental lines, parental F1 and a F2 generation defined in this order. Here the grandparental lines are called JI98 and Molle and the F1 and F2 are simply called F1 and F2, respectively.

**Line 4**: Names given to males and females. Because QTL-Express is used not only for analysis of hermaphrodite plants, but also for animal analysis, it was designed to identify male and female effects. Here males are M and females F.

**Line 5**: Symbol used for missing genotype data. In this case I used '0'.

**Line 6 onwards**: Data for each individual in turn. Contains in order:

**Individual ID** - individuals were named from 1 to 107. For F2 individuals this ID corresponded to an individual with trait data in the phenotype file.

**Sire ID** - the ID of the sire, unless the individual is one of grandparental lines in which case is set at 0 for unknown. If this is a known individual, it will have its own individual record elsewhere in the file. Members of the F2 *Antirrhinum* population, however, did not have separate male and female parents, so in this space I named the sire ID as F1_m.

**Dam ID** - As for sire. Here given as F1_f

**Sex** - Code as defined in line 4. Again, because the F2 of *Antirrhinum* involved hermaphrodites, all individuals were given as male.

**Line or generation** - As defined in line 3. In this study the line was F2.

**Marker genotypes** - Two values for the two alleles of each marker locus. Markers are given in the same order as the list of marker names. The alleles from JI98 were denoted as 1 and the *A. molle* alleles as 2. Therefore a JI98 homozygote was 1 1, a plant homozygous for the *A. molle* allele was 2 2 and a heterozygote 1 2 or 2 1. For dominant markers, the genotype of a plant carrying a JI98 allele was given as 1 0, and for a plant carrying an *A. molle* allele was given as 2 0.

## Map information

The map data file included information about the markers in each linkage group and the distances between them.

```
9
1
LG1    28     1
11CACM133    2      11AGAJ185    5      11CACM185
LG2    26     1
150    9      11AGAJ089    10     11AGAJ283    1      14AGCM203
LG3    24     1
11CACJ098    6      STY    6      12AGCJ213    2      11AGGJ135
```

**Line 1**: Number of linkage groups - in this case 9.

**Line 2**: Interval for calculation of genotype probabilities and coefficients in cM. In this case 1 cM. Genotype probabilities and coefficients are used by QTL Express in regression of trait values onto genotypes.

**Line 3**: Name of the first chromosome, the number of markers on the first chromosome and whether the map is the same for both sexes (1) or not (2). Because the map was averaged for pollen and ovules (1) was used.

**Line 4**: For the first linkage group, the marker names in order separated by the distances between them in cM. Marker names must be the same as in the genotype file.

Lines 3 and 4 are repeated for each chromosome.

## Phenotype data

The following is an example of a phenotype data file.

```
0 0 2
L-Area L-Perimeter
-999
1 947 155
2 2761 324
3 1297 170
4 858 154
5 919 150
6 530 106
```

**Line 1:** The number of fixed effects (factors), covariates and traits, respectively. No fixed effects or covariates were used in my analyses. In this example there are two traits.

**Line 2**: The name of each fixed effect, covariate and trait. Because there are no fixed effects or covariates, the two names refer to the two

traits.

**Line 3**: The code for missing trait value. Traits values maybe be missing, but all individuals must have data. I used the -999, which did not occur in any of the real trait values.

**Line 4** : Individual ID (as in the genotype file), and the values for fixed effects, covariates and traits for that individual, in the order in which they are given in line 4.

## QTL Analysis of the F2 Population

This method was developed for F2 populations produced from inbred lines by Haley and Knott (1992). The analysis includes two steps. First data on marker positions are put together with actual marker genotypes and used to calculate probabilities of individuals inheriting 0, 1 or 2 alleles from each of the two founder lines at positions, specified in line 2 of the map file, throughout the genome and the parent-of-origin probability of the alleles. These probabilities are combined into "coefficients" that can be used to look at marker information content or marker segregation distortion. In the second step, the phenotypic data is regressed onto these coefficients. The simple nature of the regression approach means it is possible to fit various genetic and environmental models, such as one or two linked QTL, additive and dominance effects of QTL, effects of environmental factors (fixed effects)while maintaining computational efficiency.

The results are displayed in two steps. Firstly a summary is made of the population structure in terms of the number of families and number of progeny and the characteristics of the genetic markers in terms of number of alleles, and information content to estimate genetic parameters (called 'a' and 'd', for additive and dominance effect). Secondly, the results of the chosen QTL analysis are displayed, including the estimate of the model parameters (additive and dominance effect), the fitted fixed effects and the sums of squares of the full and reduced models (Haley *et al.*, 1994).

My F2 population did not have fixed effects because, for example, I could not identify the parental origin of alleles (the parents of a heterozygous (1 2) F2 individual are the same (1 2) F1 heterozygote).

The additive effect (a) is modelled as half of the mean difference between the two homozygotes at the QTL,

i.e., $a = \frac{(QQ_{(P1)} - qq_{(P2)})}{2}$, $QQ$ is genotype value of parent 1, $qq$ is genotype value of parent 2, so that a positive value of $a$ indicates that the allele increasing the trait value originates from parent 1.

The dominance effect ($d$) is defined as $d = Qq - \frac{1}{2}(QQ + qq)$, where $Qq$ is the mean heterozygote trait value. A positive value indicates that the heterozygote has a larger than the midparental value (Knott *et al.* 1998). For a locus with dominant alleles the magnitude of $d$ is equal to $a$.

The relationships between genetic and environmental components of phenotypic variance is shown in Figure 2.4.2; together with the additive and dominance contributions to genetic variance.

The permutation test (Churchill and Doerge, 1994) is implemented by permuting the genetic coefficients for a chosen chromosome of an individual over trait values. In this way, the estimates of the fixed effects are unaffected while the relationship between the genotypes and phenotypes are shuffled. QTL analysis of these permutations (usually 1,000) therefore suggested the likelihood of detecting a QTL at a particular position by chance.

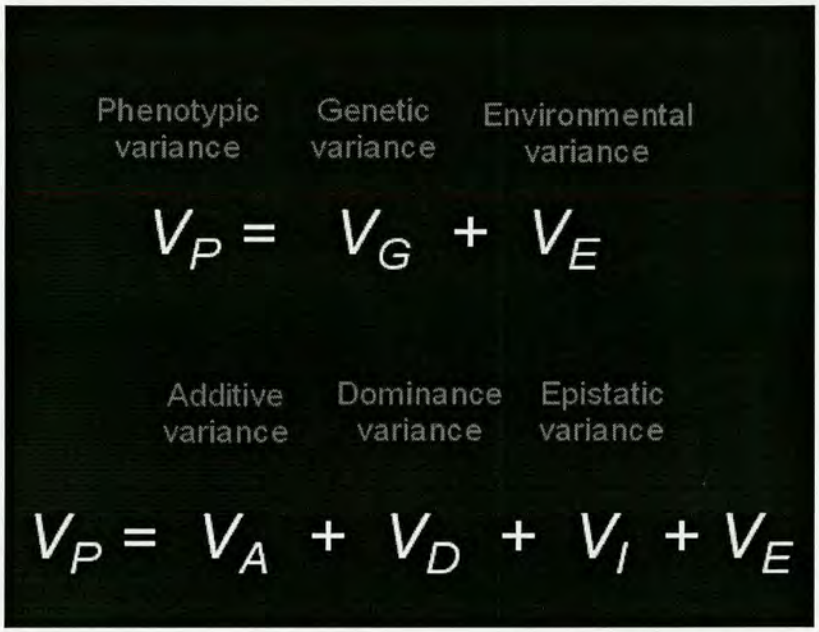



**Figure 2.4.2 The relationship between genetic and environmental effects on phenotypic variance.** The total variance can be described as the sum of genetic and environmental variance. Genetic variance can itself be considered the sum of additive and dominance effects with the effects of epistatic interactions (i.e. non-additive interactions between loci).

All QTL analyses took advantage of the ability to fix the effects of loci in QTL-express. Therefore a step-wise regression method was used. This involved scanning the genome for significant QTLs affecting a trait. The most likely position of the most likely QTL was fixed and the QTL scan repeated until no more significant QTLs could be detected. Significance was judged by permutation analysis, involving 1,000 permutations of genotypes within each chromosome. Having fixed all significant QTLs as cofactors, the effect of each QTL was estimated in turn by unfixing it while keeping all other significant QTLs remaining as cofactors. The values of *a* and *d* and the likelihood for each QTL were estimated directly by QTL-express under these conditions. Although this method allows detection of linked QTL, it often gives different estimates of the mean value of each trait, particularly for linked QTLs.

Likelihood of a QTL at its most likely position was expressed as an F-value (Fischer, 1925) and as a LOD score. The percentage of variance explained by each QTL was calculated from the residual variance estimated by regression in QTL-express when the QTL was fitted when all other significant QTLs were fixed ($RSS_{unfixed}$; RSS is residual sum of squares from

the regression analysis), the estimated residual variance when this QTL was also fixed ($RSS_{fixed}$) and the total variance with no QTL fixed ($RSS_{total}$). The percentage variance explained was calculated as ((($RSS_{unfixed}$ - $RSS_{fixed}$)/ $RSS_{total}$) x 100). In general the most likely QTL also had the highest value of variance explained.

The confidence interval for the position of a QTL was estimated using the assumption that the interval in which the LOD score dropped by a value of 1 from its maximum value corresonded to the 99% confidence interval and a 2-LOD drop corresponded to the 95% confidence interval (Knott *et al.* 1998). Because QTL-express plotted only F values against chromosome position for each QTL, LOD values were calculated from the F-value plotting data returned by QTL-express using the formula

$$F = \frac{n}{\Delta n}\left(10^{\frac{2}{n}(LOD_{MAX-1})} - 1\right)$$, where $n$ is the total degrees of freedom without the QTL fitted, $\Delta n$ is the number of degrees of freedom lost in fitting the QTL and $LOD_{MAX-1}$ is 1 below the maximum LOD score.

## QTL Analysis of F3 Population

F3 population from different F1 individuals (Edinburgh & Germany line) were grow together under the same condition in Edinburgh. About 850 and 770 individuals were grown from the Edinburgh and Germany lines, respectively. Both F3 were scored for major vegetative (leaf) variance for QTLs mapping with the combined linkage map from both Edinburgh and Germany F2 genotype (Figure 2.4.3, also see RESULTS). In order to do so, for each trait, F3 individual data from each line were collected and calculated to get the mean value of each trait. This allowed use of the same genotype and map data to QTL-express as for the F2 populations.

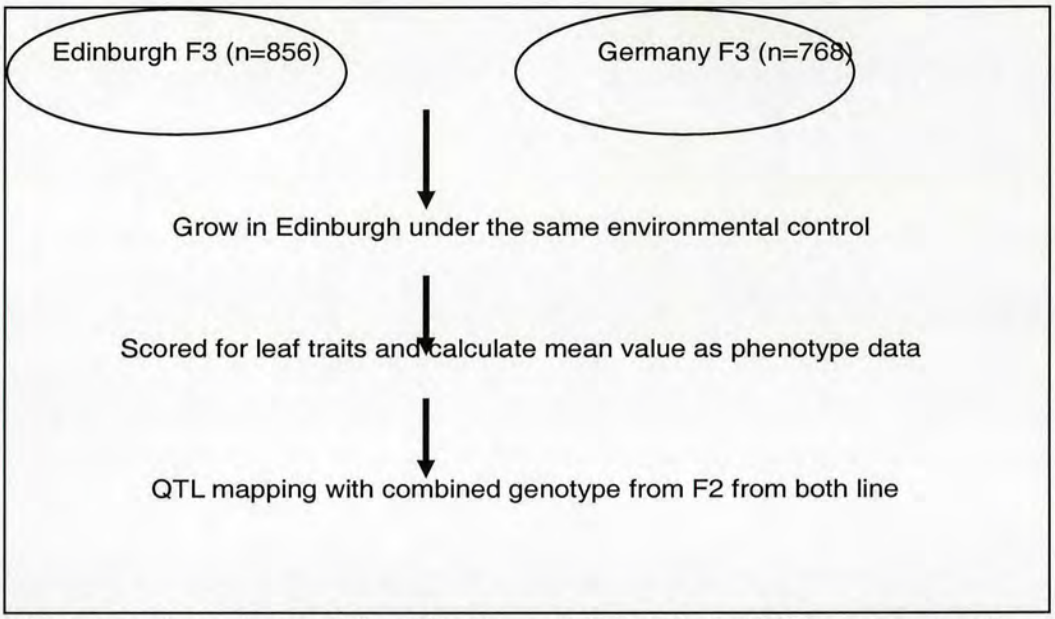



**Figure 2.4.3 A graphic display of how F3 families were used.**

## 2.5. Heritability

Narrow sense heritability, $h^2$, was calculated for the F3 population as

$$h^2 = \frac{Var(A)}{Var(P)}$$

Where ***Var(A)*** is the additive genetic variance and ***Var(P)*** is the phenotypic variance. Heritability indicates the proportion of phenotypic variance in a population that is attributable to genetic variation among individuals. Members of the same F3 family, derived from the same F2 plant, were assumed to be genetically identical, therefore variance within families was used as an estimate of the environmental variance, *Var(E)*. The additive genetic variance was calculated as *Var(P)-Var(E)*.

## 2.6. Identification of Loci involved in Hair Density

Hair density segregated in the F2 population of *A. majus* x *A. molle*. In the F3 population of the Edinburgh line, two very hairy individuals (as hairy as *A. molle*) were found. These two individuals were back-crossed to *A. majus* as female parent, which was proposed to be homozygous for an inhibitor of hair formation. These back-cross plants were self-

pollinated to test whether hairiness segregated with candidate loci in the next generation.

## Identifying Candidate Hair Genes

### • Genomic DNA Preparation

Genomic DNA was extracted from young leaves with the method described by Doyle and Doyle (1987). About 0.5 g of fresh leaves was collected from each individual and frozen and ground in liquid nitrogen. Extraction buffer (1 ml) was added and the mixture incubated in a microfuge tube at 65ºC for 20-30 min. One half volume of chloroform (0.5 ml) was then added, mixed by vortexing and the tubes centrifuged at 13,000 *g* for 10 min. The supernatant was recovered to a clean tube and nucleic acids precipitated from it with two-thirds the volume (0.67 ml) of isopropanol at room temperature. Nucleic acids were recovered by centrifugation, washed in 70% ethanol and dissolved in 50 µl of TE containing 10 µg/ml RNase A.

| **Extraction buffer** | |
|---|---|
| 100 mM Tris, pH 8.0 | 10.0 ml 1.0M |
| 1.4 M NaCl | 28.0 ml 5.0M |
| 20 mM EDTA | 4.0 ml 0.5M |
| 2% CTAB | 2.0 g |
| 0.2% β-MCE (added just before use) | 2.0 µl/ml |
| $dH_2O$ | to 100.0 ml |

### • Mapping of the *MIXTA-like1* (*ML1*) Gene

## PCR and Amplification

Members of the *MIXTA-like* family of *MYB* genes were candidates for loci controlling hair density. Therefore plants segregating for hair density were genotyped at *MIXTA-like* loci. Amplification of *ML1* used reverse primer, *MixL1R3* combined with forward primers, *ML1B#17*, specific for the *A. molle* allele and *ML1B#20*, able to amplify only the *A. majus* allele. The sequences are as follow:

***ML1R3***

5′ -AAA GGC ATG AAG TTG GAT AAT AGC-3′

***ML1B#17F*** (*A. molle* allele)

5′ - TCT CGT TGC AAA ATA CTC TAG-3′

***ML1B#20F*** (*A. majus* allele)

5′ -TGC AAA ATA CTC CTT CTT CTA C-3′

The optimum annealing temperature for the primers was determined to be 57°C. PCR was carried out using the components and conditions shown below. Reaction products (~400 bp) were later analysed in 1% agarose gels.

**PCR reaction components**

| | |
|---|---|
| Genomic DNA | 2.0 µl |
| 10x PCR buffer(2 mM $MgCl_2$) | 1.0 µl |
| dNTP 10 Mm | 0.2 µl |
| Mixta-like 1R3 10 µM | 0.2 µl |
| Mixta-like1B #20 10 µM | 0.2 µl |
| or | |
| Mixta-like1B #17 10 µM | 0.2 µl |
| Taq 1 U/µl | 0.25 µl |
| $dH_2O$ | 6.15 µl |
| TOTAL | 10.0 µl |

**Amplification cycling conditions**

| | | |
|---|---|---|
| 94°C | 4.0 min | |
| 94°C | 45 s | |
| 57°C | 1.0 min | |
| 72°C | 1.5 min | } 35 times |
| 72°C | 7.0 min | |

- **Mapping of the *MIXTA-like 2 (ML2)* Gene**

## PCR and Amplification

Amplification of *ML2* was carried out using forward primer, *Mix CF*, and reverse primer, *MixL2R*. Primer sequences are as follow:

***Mix CF***

5′ -AGA ACA TGG YCA YGG AAR CTG G-3′ where Y = C or T and R = A or G.

***MixL2R***

5′ -CTC GTT GTC CGT TCG TTT CGG T-3′

The optimum annealing temperature had been determined to be 70°C (Emma Persson, personal communication). PCR was carried out using the components and conditions as shown below. Reaction products were analysed on 1% agarose gels to test whether amplification had been successful.

**PCR reaction components**

| | |
|---|---|
| Genomic DNA | 2.0 µl |
| 10x PCR buffer(2 mM $MgCl_2$) | 1.0 µl |
| dNTP 10 mM | 0.2 µl |
| Mix CF 10 µM | 0.2 µl |
| Mix L2R 10 µM | 0.2 µl |
| Taq 1 U/µl | 0.25 µl |
| $dH_2O$ | 6.15 µl |
| TOTAL | 10.0 µl |

**Amplification cycling conditions**

| | |
|---|---|
| 94°C | 2.0 min |
| 94°C | 20 s |
| 70°C | 20 s |
| 72°C | 2.0 min |
| 72°C | 10.0 min |

} 35 times

## Digestion

The amplified products were digested with 0.5 µl(1 unit)of *Nla* III enzyme using 5.0 µl reaction products mixed with 1.0 µl 10x NEB4 buffer and 4.5 µl $dH_2O$. Digestion was then carried for 2 hours at 37°C. All the digested products were run on a 3% agarose gel to identify polymorphisms.

- ## Mapping of the *CYCLOIDEA (CYC)* Gene

The *CYC* gene is linked to *ML2*. Amplification of *CYC* was carried out using forward primer, *CYC F* and reverse primer, *CYC R*. The sequences are as follows:

***CYC F***

5' -TCC TCC CTT CAC TCT CGC GC-3'

***CYC R***

5' -TGG CGC ATA GCT GGT TCG AC-3'

The optimum annealing temperature for *CYC* primers had been determined as 55°C. PCR was carried out using the components and conditions shown below. Reaction products were analysed on 1% agarose gels.

**PCR reaction components**

| | |
|---|---|
| Genomic DNA | 2.0 µl |
| 10x PCR buffer(2 mM $MgCl_2$) | 1.0 µl |
| dNTP 10 mM | 0.2 µl |
| *CYC F* 10 µM | 0.2 µl |
| *CYC R* 10 µM | 0.2 µl |
| Taq polymerase 1 U/µl | 0.25 µl |
| $dH_2O$ | 6.15 µl |
| TOTAL | 10.0 µl |

**Amplification cycling conditions**

| | |
|---|---|
| 94°C | 4.0 min |
| 94°C | 45 s |
| 55°C | 45 s |
| 72°C | 1.5 min |
| 72°C | 7.0 min |

} 35 times

## Digestion

The amplified products were digested with 0.5 µl (1 unit) of *Rsa* I with 5.0 µl reaction products mixed with 1.0 µl of 10x NEB1 buffer and 4.5 µl $dH_2O$. Digestion was carried out for 2 hours at 37°C and the products were run out on a 3% agarose gel to detect a polymorphism.

- **Mapping of the *MIXTA-like3* (*ML3*) Gene**

## PCR amplification

Amplification of *MIXTA-like3* used forward primer, *ML3F3* and reverse primer, *ML3R3*. The primer sequences are as follow:

***ML3R3***

5' -AAA CAC TCG GAA AAT AGG GGA ATC-3'

***MixL3F3***

5' -TTA GCC AGA CAA TCA AAC TCC A

The primers were tested for best annealing temperature and used with the components and conditions shown below. Reaction products were analysed on 1% agarose gels.

**PCR reaction components**

| | |
|---|---|
| Genomic DNA | 2.0 µl |
| 10x PCR buffer(2 mM $MgCl_2$) | 1.0 µl |
| dNTP 10 Mm | 0.2 µl |
| ML3R3 10 Mm | 0.2 µl |
| ML3F3 10 Mm | 0.2 µl |
| Taq 1 U/µl | 0.25 µl |
| $dH_2O$ | 6.15 µl |
| TOTAL | 10.0 µl |

**Amplification cycling conditions**

| | |
|---|---|
| 94°C | 2.0 min |
| 94°C | 15 s |
| 50°C | 20 s |
| 72°C | 45 s |
| 72°C | 5.0 min |

} 35 times

## Digestion

The amplified products were digested with 0.1 µl *Aci* I enzyme using 4.0 µl reaction products mixed with 1.0 µl 10x NEB4 buffer and 4.9 µl $dH_2O$. Digestion was then carried out at 37°C for two hours. The digested products were run out on a 3% agarose gel to detect a polymorphism.

# Chapter 3: RESULTS

## PHENOTYPICAL ANALYSIS

### 3.1 Correlation Analysis

An F2 population from *A. majus* x *A. molle* was generated in order to detect genes responsible for differences between two parental species and a number of different vegetative and reproductive traits were recorded for analysis. The correlation between traits was analysed, with the view that traits under the same genetic or environmental control would be correlated.

The correlations among vegetative and reproductive traits were estimated by the correlation coefficient,

$$r_{xy} = \frac{\mathrm{cov}(x,y)}{s_x s_y} \approx \frac{\Sigma(X-\overline{X})(Y-\overline{Y})}{\sqrt{\Sigma(X-\overline{X})^2 \Sigma(Y-\overline{Y})^2}}$$

where cov(x,y) is the covariance means for traits x and y, *Sx* and *Sy* are the square roots of the respective among variance components for each trait (Robertson, 1959). The significance of each correlation can be determined using a t-test after a z-transformation of the correlation coefficient as described by Sokal and Rohlf (1981). As I studied a large number of individuals ($n$ = 107) the correlation coefficient can be estimated by the second formula, where $X$ and $Y$ are the individual values for two traits and $\overline{X}$ and $\overline{Y}$ are their mean values. In this study, 63 traits were analysed and a correlation matrix built to display correlations between them. A total of 1,953 correlations were estimated. Eighty seven appeared highly correlated ($|r| \geqq 0.6$), whilst 208 showed mid-correlation ($0.3 \leqq |r| < 0.6$).

The phenotypic traits were divided into ten groups as shown below; the correlations were estimated within and between these groups.

1 Whole leaf traits (Traits 1-7)

2 Upper leaf epidermal cell traits (Traits 8-14)

3 Lower leaf epidermal cell traits (Traits 15-21)

4 Whole flower traits (Traits 22-32)

5 Dorsal petal traits (Traits 33.39)

6 Abaxial petal epidermal cell traits (Traits 40-46)

7 Other size and shape traits (Traits 47-51)

8 Flower pigmentation (Traits 52-54)

9 Abaxial leaf hair density and morphology (Traits 55-59)

10 Adaxial leaf hair density and morphology (Traits 60-63)

## Correlations within Groups

Correlations were estimated within each group (i.e. between leaf traits or between floral traits), in order to find out if traits within groups are affected by the same factors. Traits in Group 2 (upper leaf epidermal cell traits) were most highly correlated (61.9%, $|r| \geq 0.6$). Four high correlations (40.0% of the total) were found within Group 7 (other size and shape traits), significant correlations were found for the comparisons within Group 1 (whole leaf traits), 8 (flower pigment concentration) and 10 (adaxial leaf hair density and morphology). Group 3, 5, and 6 had 28.6% high correlations within the group. Unexpectedly, 11 flower traits (Group 4) had few high correlations (1.8%) - the lowest percentage compared to other groups. This suggests that flower traits are under control of different genes (Figure 3.1).

| Group | Traits | No. of high-correlated $\|r\| \geq 0.6$ | No. of intermiddle-correlated $0.3 \leq \|r\| \square 0.6$ | No. of low-correlated $\|r\| \square$ 0.3 |
|---|---|---|---|---|
| 1 | 1-7 | 7 (33.33%) | 2 | 12 |
| 2 | 8-14 | 13 (61.90%) | 4 | 4 |
| 3 | 15-21 | 6 (28.57%) | 5 | 10 |
| 4 | 22-32 | 1 (1.82%) | 25 | 29 |
| 5 | 33.39 | 6 (28.57%) | 1 | 14 |
| 6 | 40-46 | 6 (28.57%) | 8 | 7 |
| 7 | 47-51 | 4 (40.00%) | 5 | 1 |
| 8 | 52-54 | 1 (33.33%) | 1 | 1 |
| 9 | 55-59 | 2 (20.00%) | 2 | 6 |
| 10 | 60-63 | 2 (33.33%) | 0 | 4 |

**Figure 3.1 Number of correlations with each group of traits.** High correlations were estimated as $|r| \geq 0.6$ ; mid-correlations $0.3 \leq |r| < 0.6$, and no correlation if $|r| < 0.3$. Group 2 shows the highest percentage of correlations (61.9%), followed by Group 7 (40.0%), Group 1 (33.3%), Group 8 (33.3%), Group 10 (33.3%), Group 3(28.6%), Group 5 (28.6%), Group 6 (28.6%), Group 9 (20.0%), and Group 4 (1.8%).

- **Whole leaf traits**

Within Group 1, leaf perimeter and maximum length showed the highest correlation to each other ($r$ = 0.97); leaf perimeter is also highly correlated with leaf area ($r$ = 0.95), circularity ($r$ = 0.62), and maximum breadth ($r$ = 0.67). Leaf area also showed positive correlation with leaf maximum length ($r$ = 0.93) and maximum breadth ($r$ = 0.84). Leaf maximum length also gives a high positive correlation with leaf maximum breadth ($r$ = 0.67). These correlations were not unexpected as an increase in area can reflect an increase of either length or breadth. Leaf perimeter also increases when leaf area is enlarged (Figure 3.1.1).

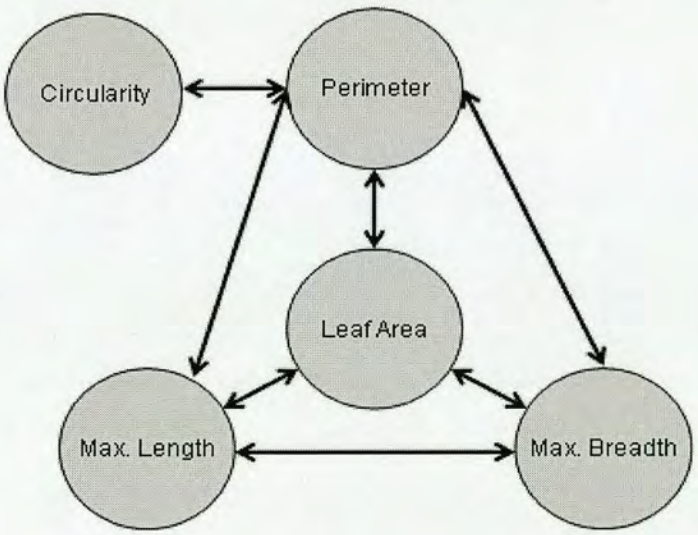



**Figure 3.1.1 The relationship of leaf traits in group one.** The traits linked with black arrows are highly correlated.

- **Adaxial leaf epidermal cell traits**

In Group 2 (adaxial leaf epidermal cell traits) cell area is highly correlated with cell perimeter, cell maximum length and breadth. It suggests that an increase in cell size (area) reflects an increase in length or breadth. Cell perimeter also increases when cell size enlarged. Cell roundness also shows a positive correlation with cell perimeter, cell length and breadth, but is not correlated with cell area ($r = -0.06$). Cell roundness estimates the similarity of the outline of a cell to a circle. It is therefore expected to be independent of cell area. The correlation between cell roundness and cell length and breadth suggests that differences in cell length and breadth are disproportionate, so that they affect the shape of the cell. The correlation between cell roundness and cell perimeter might result from cells that have a more undulating outline having a longer perimeter and being less round (Figure 3.1.2).

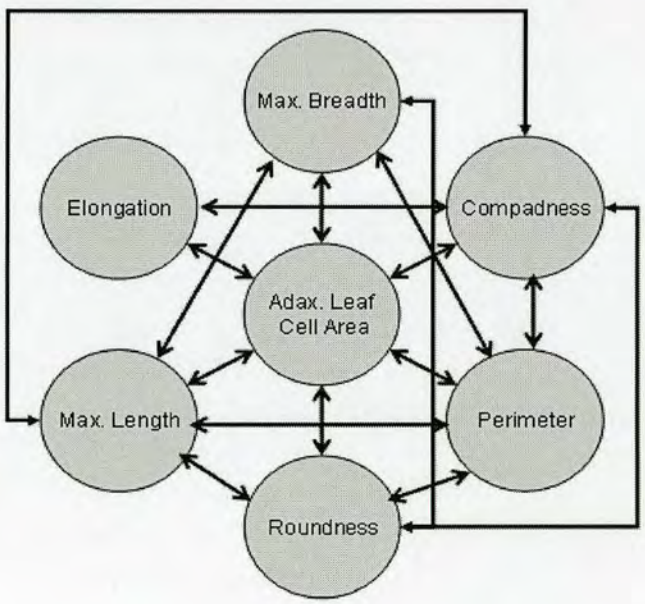



**Figure 3.1.2 the relationship between adaxial leaf cell traits.** High correlations, shown by black arrows, are found between many traits.

## • Abaxial leaf epidermal cell traits

Within Group 3 (abaxial leaf epidermal cell traits), cell area, perimeter, maximum length and maximum breadth show high correlation to each other. This suggests that an increase in abaxial cell area and perimeter can result from an increase in either cell length or cell breadth (Figure 3.1.3).

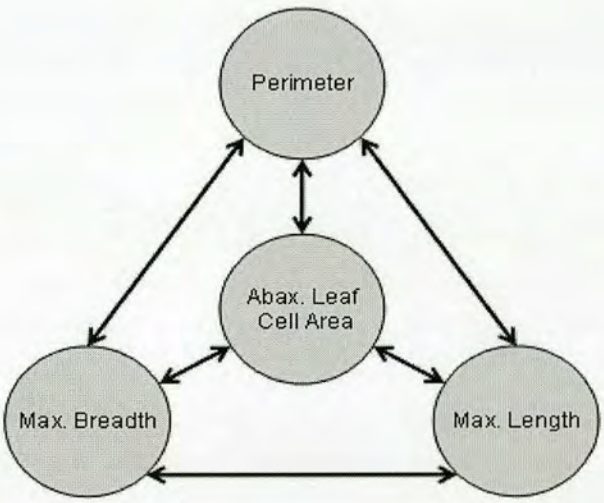



**Figure 3.1.3** Highly correlated traits for abaxial leaf cells.

## • Reproductive traits

In reproductive traits, 11 floral traits were measured and correlations calculated. Flower tube length (F2) showed a high correlation with short anther filament length (F11, $r = 0.65$) but long anther filament length (F10) did not show a similarly high correlation ($r = 0.51$). Anatomically, the end of the style (F9) is normally found between long (F10) and short (F11) anthers. Style length was therefore expected to show a high correlation with the filament length of both anthers. The results showed only mid-correlation ($r = 0.57, 0.52$). The larger flower is expected to have a stronger pedicel (F1) to support it, however the results did not show a high correlation of these traits ($-0.47 \leqslant r \leqslant 0.15$). Other traits unexpectedly showed low correlations. The width of the ventral petal lobe (F4) is not related to the width of the lower lip (F7); flower tube length (F2) and width (F3) show a low correlation ($r = 0.18$); length of the abaxial (upper) petal (F6) does not correlate with tube length ($r = 0.39$) and the height (F7) and width (F8) of the ventral petal are not significantly

correlated ($r$ = 0.18), suggesting that size of the ventral petal lobe is under different genetic control to length and width of the flower (Figure 3.1.4). Lack of significant correlations between dimensions of the flower also suggested that there was considerable variation in shape.

| | F1 | F2 | F3 | F4 | F5 | F6 | F7 | F8 | F9 | F10 | F11 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **F1** | 1 | 0.00 | -0.21 | -0.04 | -0.47 | -0.12 | -0.05 | 0.10 | 0.15 | 0.05 | -0.24 |
| **F2** | 1 | 0.18 | 0.41 | 0.30 | 0.39 | 0.13 | 0.17 | 0.52 | 0.51 | 0.65 | |
| **F3** | 1 | 0.16 | 0.41 | 0.25 | 0.08 | 0.13 | 0.24 | 0.31 | 0.33 | | |
| **F4** | 1 | 0.45 | 0.37 | 0.57 | 0.44 | 0.42 | 0.35 | 0.30 | | | |
| **F5** | 1 | 0.36 | 0.29 | 0.20 | 0.29 | 0.46 | 0.42 | | | | |
| **F6** | 1 | 0.25 | 0.34 | 0.39 | 0.46 | 0.46 | | | | | |
| **F7** | 1 | 0.18 | 0.27 | 0.17 | 0.19 | | | | | | |
| **F8** | 1 | 0.43 | 0.12 | 0.26 | | | | | | | |
| **F9** | 1 | 0.57 | 0.52 | | | | | | | | |
| **F10** | 1 | 0.51 | | | | | | | | | |
| **F11** | 1 | | | | | | | | | | |

**Figure 3.1.4 Correlation coefficient between flower traits.** Shading in pink shows that flower tube length (F2) is highly correlated with long another filament length (F11). Cells in yellow show traits with mid-correlation ($0.3 \leqslant |r| < 0.6$). **F-1:** pedicel length. **F-2:** flower tube length. **F-4:** mid-lobe width. **F-5:** gibba length. **F-6:** upper petal side length. **F-7:** lower lip width. **F-8:** height from upper petal to lower lip. **F-9:** style length. **F-11:** short anther filament length (see flower measurement in Materials and Methods).

- **Petal analysis**

Petal analysis also showed that abaxial petal area, perimeter, maximum length and breadth are all highly correlated to each other (Figure 3.1.5).

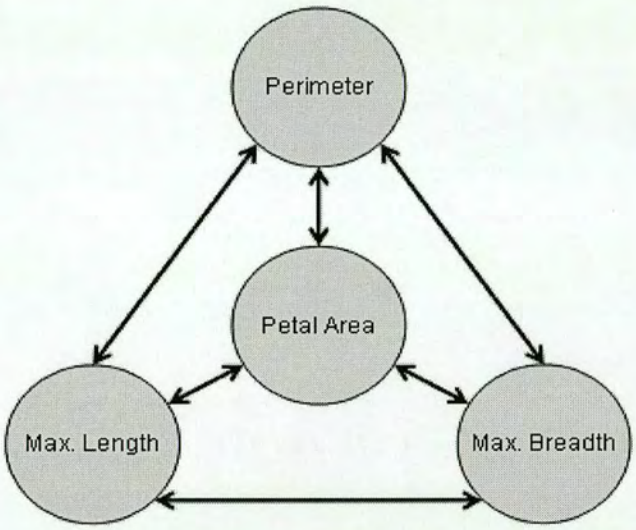



**Figure 3.1.5**
**Relationships of traits within abaxial petals.**

- **Petal cell imprints**

Traits within Group 6, abaxial petal epidermal cell traits, show that cell area is correlated to both maximum cell length and breadth. Cell perimeter is also correlated to both length and breadth. It also shows that cell roundness has positive high correlation with cell compactness, which is not unexpected because both estimate cell shape. However cell maximum length shows only mid-correlation with cell breadth ($r$ = 0.57) suggesting that cells differed in shape (Figure 3.1.6).

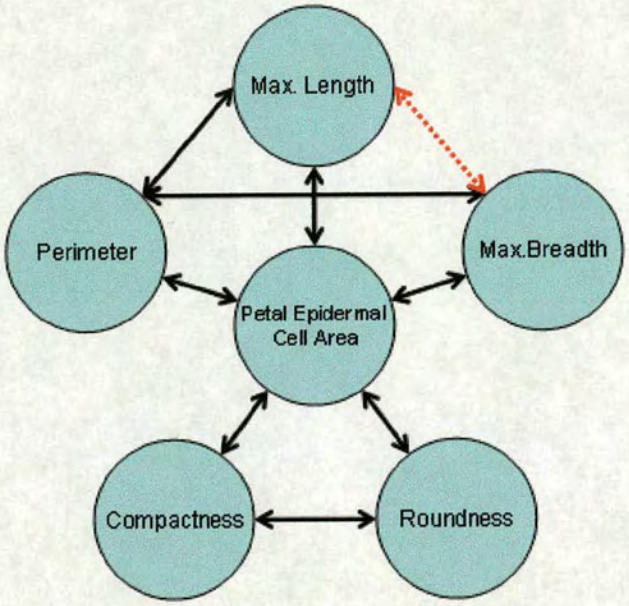



**Figure 3.1.6 Relationships of traits within abaxial petal epidermal cells.** The positive correlations are shown in black, negative correlations in red.

## • Other traits

Within Group 7, the node where the first flower formed showed a very high correlation with plant length and number of lateral branches. This suggested that taller plants have more nodes and therefore more nodes to produce side branches. Node diameter at the base of the plant is strongly correlated with plant height, suggesting that growth in height is controlled by the same factors as growth in stem width, but node diameter was not highly correlated with branch number ($r$ = 0.59). The flowering node number seems to be in mid-correlation with flowering time ($r$ = 0.43). This suggests that later flowering plants grow taller. However, the result only showed mid-correlation ($r$ = 0.53), suggesting that plants develop (produce nodes) at different rates and that this is under different genetic control to flowering time. Later flowering plants do not make more branches ($r$ = 0.35), supporting this view (Figure 3.1.7).

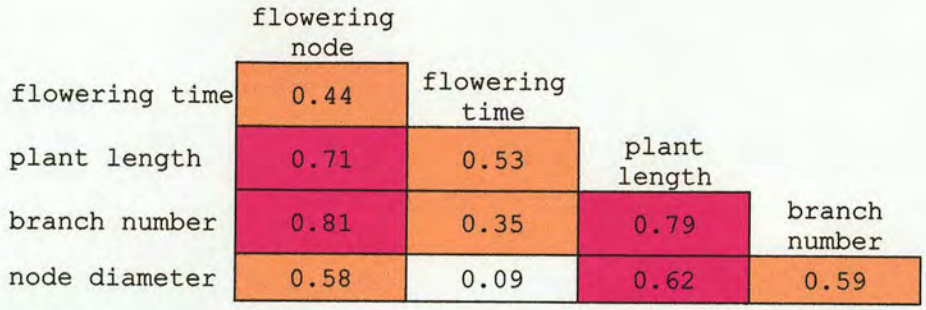



**Figure 3.1.7 Correlations between plant length, flowering node, branch number and node diameter.**

- **Anthocyanin analysis**

In pigment analysis, the concentration of anthocyanin extracted from a uniform area of the abaxial (upper) petal lobe showed a high correlation with red pigment expression judged visually ($r$ = 0.69). Yellow pigment expression, judged visually, was not correlated with anthocyanin expression.

- **Leaf trichomes**

For leaf trichomes, both abaxial and adaxial hairs per unit area are highly correlated with hair distribution over the whole leaf area. Other leaf hair traits do not show any similarly high correlations.

## Correlations between Groups

To estimate the relationships between vegetative and reproductive traits, a matrix was built to calculate the number of correlations between groups (Figure 3.1.8). Results show correlations involving Groups 9 and 10 (abaxial trichomes and adaxial trichomes). Group 9 shows correlated with all other groups except Group 8, Group 10 also shows correlations with Group 2 and Group 9 (Figure 3.1.8).

| group | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | 0 | 0 | 2 | 0 | 2 | 5 | 0 | 0 | 0 |
| 2 | 0 | | 20 | 4 | 4 | 0 | 3 | 0 | 8 | 10 |
| 3 | 0 | 0 | | 6 | 15 | 4 | 1 | 0 | 8 | 2 |
| 4 | 0 | 0 | 0 | | 8 | 0 | 4 | 0 | 8 | 3 |
| 5 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 3 | 7 |
| 6 | 0 | 0 | 0 | 0 | 0 | | 0 | 0 | 6 | 0 |
| 7 | 0 | 0 | 0 | 0 | 0 | 0 | | 8 | 7 | 0 |
| 8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | 2 | 0 |
| 9 | 2 | 7 | 1 | 14 | 6 | 4 | 1 | 0 | | 5 |
| 10 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | |

**0.3≤|r|<0.6**

**|r| ≥0.6**

**Figure 3.1.8 number of correlations between each group.** High correlations (|r| ≥0.6) are shown in blue, intermiddle correlations (0.3≤|r|<0.6) in pink.

Whole leaf area does not show any significant correlation with adaxial leaf cell area ($r$ = 0.04) or abaxial leaf cell area ($r$ = -0.04). This suggests that differences in leaf area reflect differences in cell number (and therefore cell division) and not differences in cell size (i.e. cell expansion). Abaxial and adaxial cell size show mid-correlation ($r$ = 0.48), suggesting that cells in the two surfaces of the leaf are affected by some common factors.

In abaxial (upper) flower petals, petal size is not correlated with petal length at the base where the petal lobe is joined to the flower tube (F6, $r$ = 0.29), however the petal perimeter shows a high positive correlation with petal size ($r$ = 0.95). This suggests that petal area is related to an increase in overall size rather than an increase in the petal base length. For the maximum vertical length and horizontal breadth of the petal area (on abaxial petals separated from the tube), the results show that whole petal size is related to variation in both vertical and horizontal lengths.

The abaxial petal area does not show any significant correlation with the petal epidermal cell area ($r$ = 0.08) or perimeter ($r$ = 0.06). This suggests

that the whole abaxial petal size is affected by the number of cells rather than the size of the epidermal cells.

Comparing leaf area with petal area, the two traits show very low correlation ($r$ = 0.08), suggesting that growth of petals and leaves are controlled largely independently. Adaxial leaf cell area was also not correlated with petal area ($r$ = -0.12 ), suggesting that, as in leaves, differences in petal area reflected differences in cell division, rather than cell expansion. However, the abaxial leaf cell area showed a negative correlation to petal area ($r$ = -0.45). This might reflect common genetic control of the two processes, or the action of different genes that were closely linked.

For trichomes, the density of long hairs on the abaxial leaf, showed a high positive correlation with abaxial leaf cell length and elongation, but did not seem to be related to epidermal cell area or cell breadth. This suggests that the factor controlling polar expansion of epidermal pavement cells might also operate in hair development. The result suggested that greater cell expansion could lead to larger pavement cells and longer trichomes (as *A. majus*) while weaker cell expansion caused smaller pavement cells and short trichomes (as *A. molle*). Density of long abaxial hairs (trait 51) also showed a high negative correlation with flower tube length (F2), flower tube height (F3), width of middle lobe (F4), gibba length (F5), side length of the upper petal (F6), and short anther filament length (F11)(Figure 3.1.9).

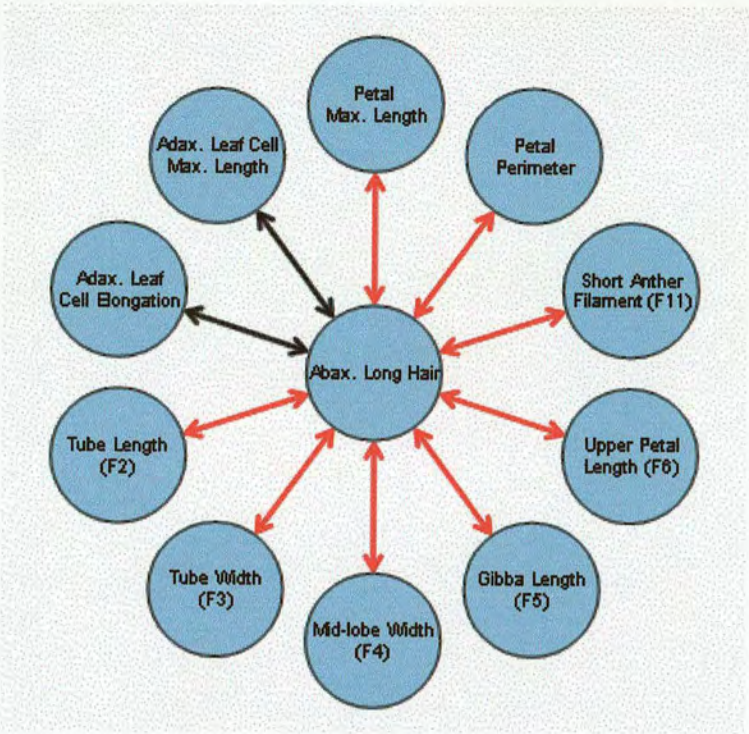



**Figure 3.1.9 correlations between abaxial trichomes density and other traits.** Positive correlations are shown in black, negative correlations are shown in red.

For adaxial leaf trichomes, over all leaf adaxial long trichome number is highly correlated with abaxial long trichome density, adaxial leaf cell area, perimeter and maximum length (Figure 3.1.10). This shows the number of long trichomes increase with leaf cell size, suggesting that similar processes might be involved in development of both. However it is not correlated with cell breadth, suggesting that polar cell growth might be involved.

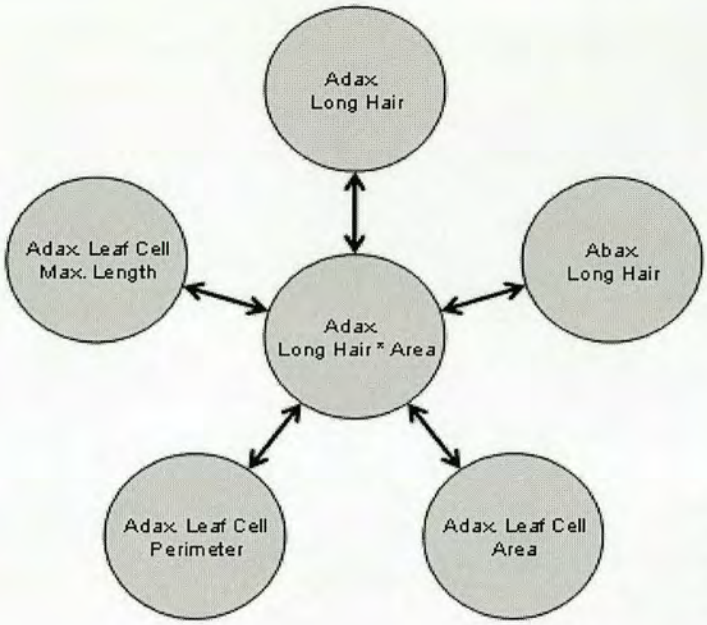



**Figure 3.1.10 correlations between overall adaxial long trichome traits with adaxial leaf cell and abaxial trichome traits.**

## 3.2 Leaf Shape Analysis

The parental species have evident differences in leaf shape and size. *A. majus* has greatly broader leaves compared to *A. molle* with smaller rounder leaves. In order to be able to analyse the differences between F2 individuals with a quantitative way, points were applied along the leaf outline.

### All points along leaf outlines are highly correlated.

There are many different ways used to describe how plant leaf shape changes in different species. Commonly, plant leaf shapes are classified qualitatively as round or pointed etc. However there are few specialized methods to describe leaf shape in a numerical way to allow analysis of how genes affect leaf shape. In order to analysis leaf shape quantitatively, I reduced the outline of each leaf in the F2 mapping population to a series of points using two different methods. Both involved points placed at landmarks (e.g. the tip of the leaf), but differed in the way that other points were placed - either by intersection with

lines spaced at a constant angle from the midrib or spaced at a constant distance around the perimeter (see Materials and Methods for further details). The mean value for each of the points in the mapping population was calculated, giving the mean shape of the leaf. Values for each point from each individual leaf were calculated as the distance to its corresponding mean point. These values were used to calculate correlations between the displacements of all points from the mean. The results, shown here for the point-placing method of Langlade *et al.*, 2005) showed that all the points are highly correlated to each other (average $r = 0.93$, Figure 3.2). This suggests that all the points should be analysed together rather than individually.

| | P1 | P2 | P3 | P4 | P5 | P6 | P7 | P8 | P9 | P10 | P11 | P12 | P13 | P14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **P1** | 1 | | | | | | | | | | | | | |
| **P2** | 0.94 | 1 | | | | | | | | | | | | |
| **P3** | 0.87 | 0.97 | 1 | | | | | | | | | | | |
| **P4** | 0.85 | 0.95 | 0.99 | 1 | | | | | | | | | | |
| **P5** | 0.92 | 0.97 | 0.96 | 0.97 | 1 | | | | | | | | | |
| **P6** | 0.96 | 0.97 | 0.93 | 0.93 | 0.98 | 1 | | | | | | | | |
| **P7** | 0.97 | 0.95 | 0.90 | 0.89 | 0.95 | 0.99 | 1 | | | | | | | |
| **P8** | 0.96 | 0.97 | 0.93 | 0.92 | 0.96 | 0.99 | 0.99 | 1 | | | | | | |
| **P9** | 0.92 | 0.95 | 0.94 | 0.94 | 0.97 | 0.97 | 0.95 | 0.98 | 1 | | | | | |
| **P10** | 0.85 | 0.91 | 0.93 | 0.94 | 0.94 | 0.92 | 0.89 | 0.93 | 0.97 | 1 | | | | |
| **P11** | 0.84 | 0.91 | 0.94 | 0.94 | 0.93 | 0.92 | 0.90 | 0.93 | 0.96 | 0.98 | 1 | | | |
| **P12** | 0.92 | 0.95 | 0.93 | 0.92 | 0.96 | 0.97 | 0.96 | 0.97 | 0.97 | 0.95 | 0.97 | 1 | | |
| **P13** | 0.98 | 0.92 | 0.84 | 0.84 | 0.92 | 0.96 | 0.97 | 0.96 | 0.92 | 0.85 | 0.85 | 0.93 | 1 | |
| **P14** | 0.92 | 0.86 | 0.80 | 0.81 | 0.89 | 0.92 | 0.91 | 0.91 | 0.89 | 0.82 | 0.79 | 0.86 | 0.92 | 1 |

**Figure 3.2 the correlation matrix for points on the leaf outline.** The correlation matrix shows that the variations in the position of all points are highly correlated to each other ($r \geqslant 0.6$).

## Principal Component Analysis

Principal component analysis (PCA) is a multivariate statistical analysis technique that transforms a data set with large numbers of highly correlated variables into a new set of uncorrelated variables called principal components (PCs). The PCs are ordered in a way that the first

components explain most of the variation within the data set. This variation is expressed in terms of the PCs rather than in terms of the original variables, thus allowing the essential aspects of the data to be represented using a space of lower dimension. The number of PCs required to capture most of the variation in the data set depends on the characteristics of the particular data set (Ramachandran, 2005). Where there is considerable covariance, as detected for points on the outline of the leaf, most of the variance can be captured by relatively few PCs. For PCA I used two different methods to represent the outline of leaves as a series of points (see Materials and Methods). Although the computation method that I developed was able to describe the position of points for PCA and identified PCAs that broadly captured the same variance as Nicolas Langlade's method, it proved unsuitable for describing the leaf shape and size for individual leaves in terms of the major principal component, because of computational problems. Therefore all descriptions of leaf shape and size variation were done using the method of Langlade *et al.*, 2005)

## Variances of each component

The principal component analysis was done in two different shape models. The first used a model made from leaves of the F2 population of *A. majus* x *A. molle* ('molle data in molle model'), the second used the leaf shape and size data for the *A. majus* x *A molle* F2 population in a shape model produced for an F2 population of *A. majus* x *A. charidemi* ('molle data in charidemi model'), which had been used to describe shape variation in this population (Langlade *et al.*, 2005). Using the same shape model for two different populations allows comparison of the effects of genes responsible for differences between the three species.

### • *A. molle* data in the molle model

Four PCs in the molle model accounted for 96.3% of the total variance in leaf shape and size in the F2 mapping population (Table 3.2; Figure 3.2). PC1 captures mainly variance in whole leaf size and explains 86% of the total variance. PC 2 captures 4.25% of the total variance, representing mainly shape changes. PC3 describes about 4% of the total variance and mainly describes differences in the angle of the petiole to the leaf blade

axis. The final major PC, capturing 1.83% of the total variance represented mainly petiole length changes relative to length of the leaf blade.

**Table 3.2 Eigen values for principal components in molle model.**

**molle data in molle model**

| PC | variance explained (%) |
|---|---|
| PC1 (size) | 86.15 |
| PC2 (shape) | 4.25 |
| PC3 (petiole angle) | 4.08 |
| PC4 (petiole length) | 1.83 |
| Total | 96.31 |

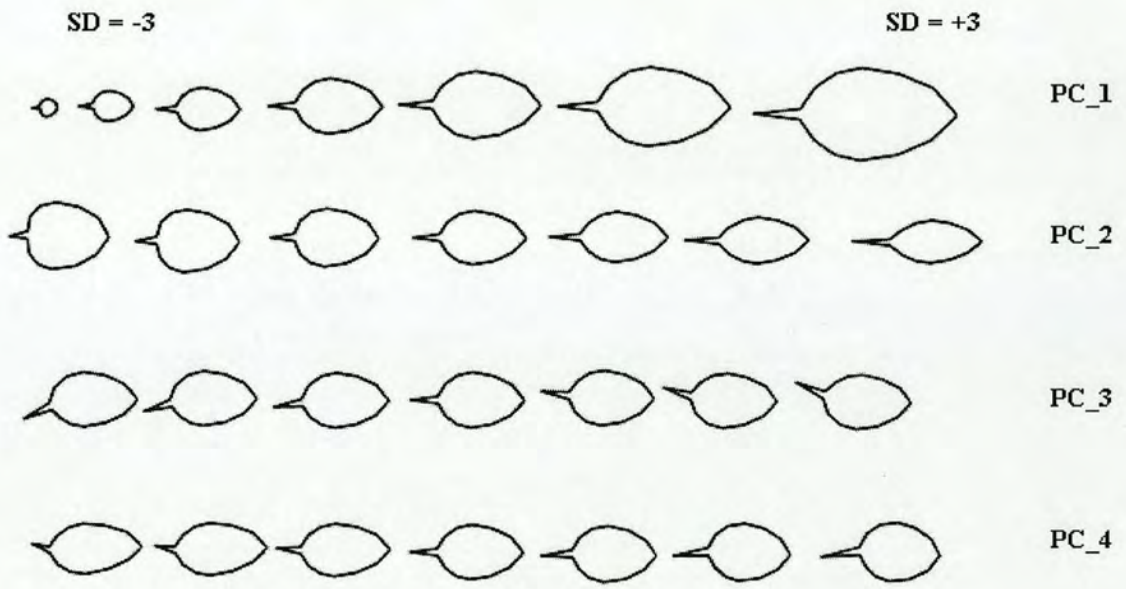



**Figure 3.2 The variation described by different principal components relative to the mean leaf shape and size in the molle model.** The mean shape of the leaves in the *A. majus* x *A. molle* F2 mapping population is shown in the centre, with the effects of varying each PC from +3SD to -3SD on each side. PC1 represents major changes in leaf size, PC2 in leaf shape, PC3 in the angle between petiole and blade and PC 4 in the relative length of the petiole.

PC3, which represented differences in the angle between the petiole and midrib of the leaf blade, did not have a significant genetic cause (see below), because it was likely to reflect differences in the way that leaves were fixed to paper for scanning. If PC3 is not considered, most (92%) of the variance in leaf shape and size is described by three PCs - PC1, PC2 and PC4. The range of shapes and sizes represented by these three PCs can therefore be represented as a 3-D space with each PC as one of the orthogonal axes (Figure 3.2.1). Nicolas Langlade had scanned leaves from populations of different Antirrhinum species and constructed points models for these (see Material and Methods for an explanation of points files). Using these data, I could capture most of the shape and size variation within the species and therefore plot the leaf shape and sizes for different species within the space defined by PC1, 2 and 4 (Fig. 3.2.1). The leaves of each species formed a cloud of points in this space, which was represented by an elipsoid. In many cases the elipsoids overlapped and together the species formed almost a continuum in the space, suggesting that the variation captured by the PCs from the molle model was typical of variation in the genus as a whole.

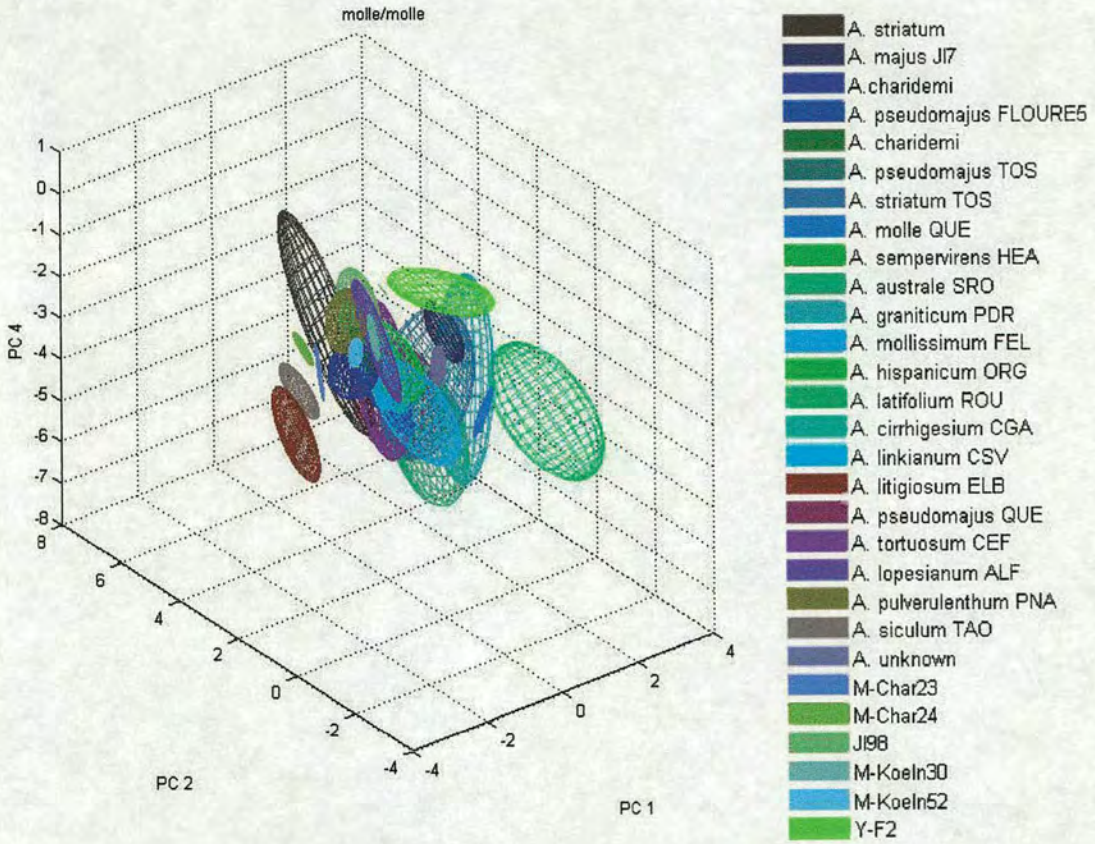



**Figure 3.2.1 Size and shape of leaves from 20 *Antirrhinum* species captured by the allometric model.** Representation of each species as a cloud in allometric space based on the $F_2$ between *A. majus* and *A. molle*. Each ellipsoid is based on leaf outlines from 2-14 individuals from each species.

Figure 3.2.1 (Continued.)

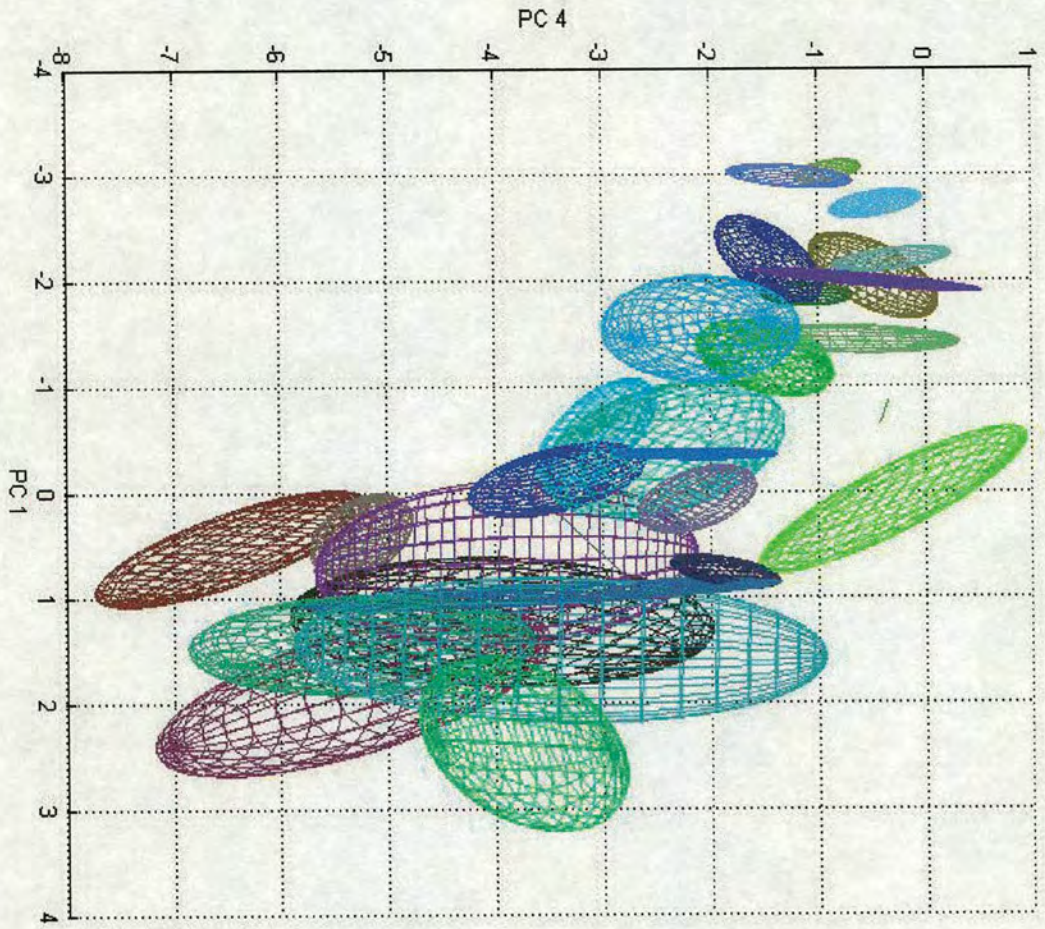



**Figure 3.2.1 (Continued.)**

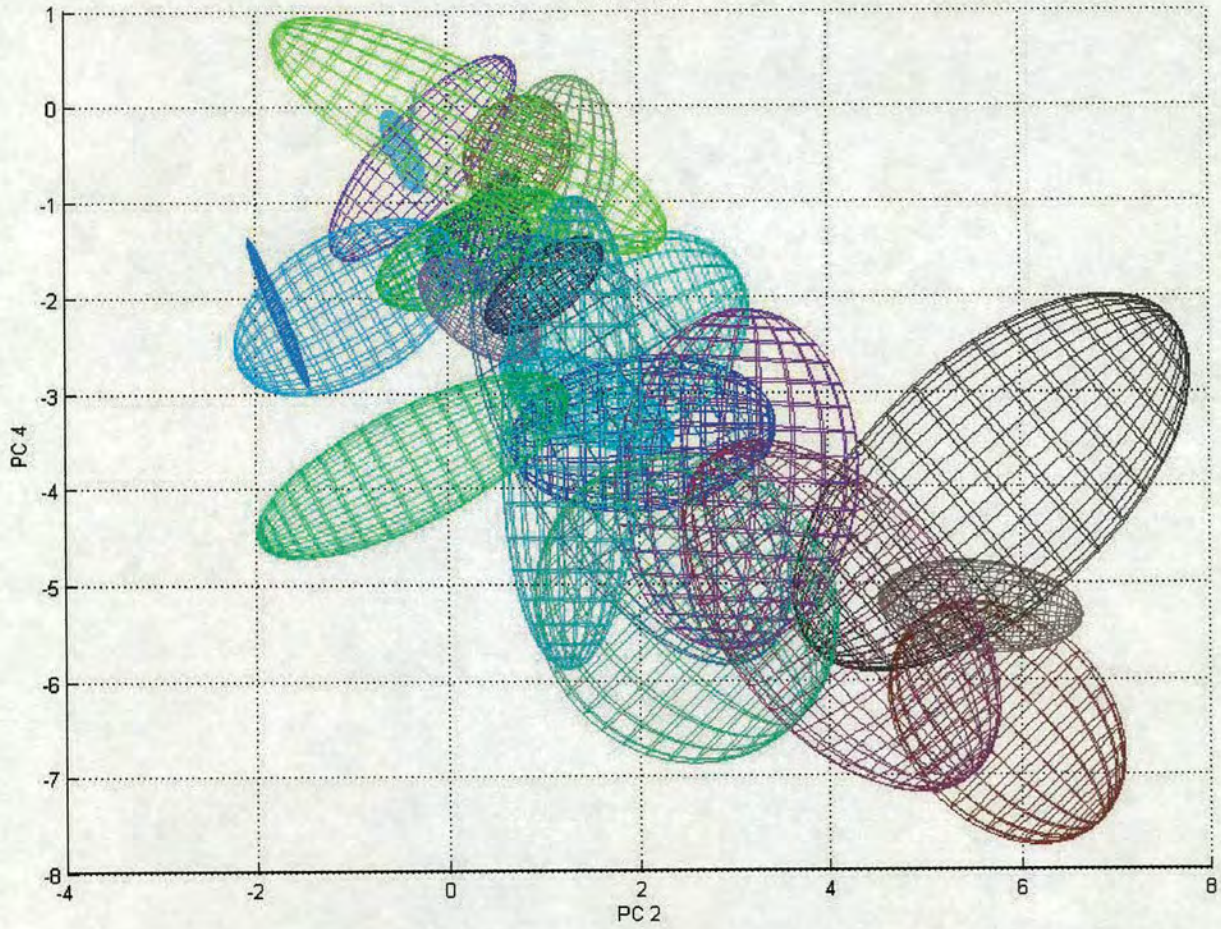



**Figure 3.2.1 (Continued.)**

## • *A. molle* data in the charidemi model

Principal component analysis of an F2 *of A. majus x A. charidemi* had revealed five PCs that described most of the variance in leaf shape and size in this population. PC1 described mainly variance in whole leaf size, PC2 mainly in leaf shape, PC3 and PC5 mainly relative petiole length, whilst PC4 mainly described the angle of the petiole relative to the blade(Langlade *et al.*, 2005; Figure 3.2.2). The shape model for this population (charidemi model) was able to describe 96.62% of the variance within the *A. majus* x *A. molle* F2 population (Table 3.2.1).

**Table 3.2.1 Eigenvalues for the *A. majus* x *A. molle* F2 population analysed with the charidemi shape model**

| principle component | variance explained(%) |
|---|---|
| PC1 (size) | 66.14 |
| PC2 (shape) | 20.38 |
| PC3(petiole length) | 4.80 |
| PC4(petiole angle) | 3.15 |
| PC5 (petiole length) | 2.16 |
| Total | 96.62 |

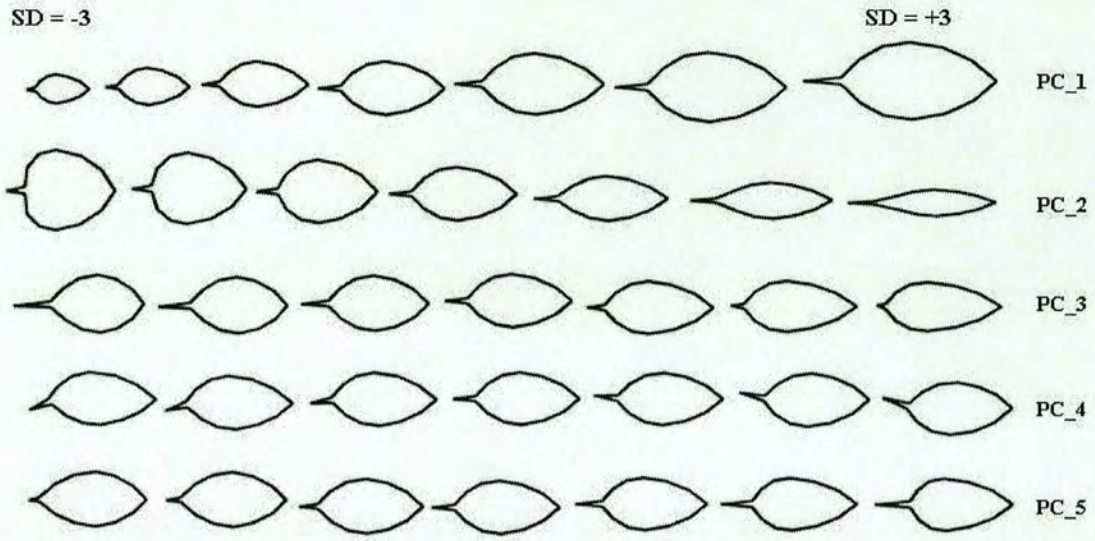



**Figure 3.2.2 Leaf shape and size differences described by different PCs of the charidemi model.** The mean leaf shape and size is shown in the centre with the effects of changing each PC from + 3SD to -3SD in steps of 1 SD. PC1 describes mainly changes in leaf size, PC2 in leaf shape, PC3 and PC5 in petiole length and leaf shape, and PC4 shows changes in petiole angle.

The first three PCs of the charidemi model had also been used to describe a space in which leaves of different Antirrhinum species could be plotted. As in the molle model, the species broadly formed a continuum in this space (Figure 3.2.3).

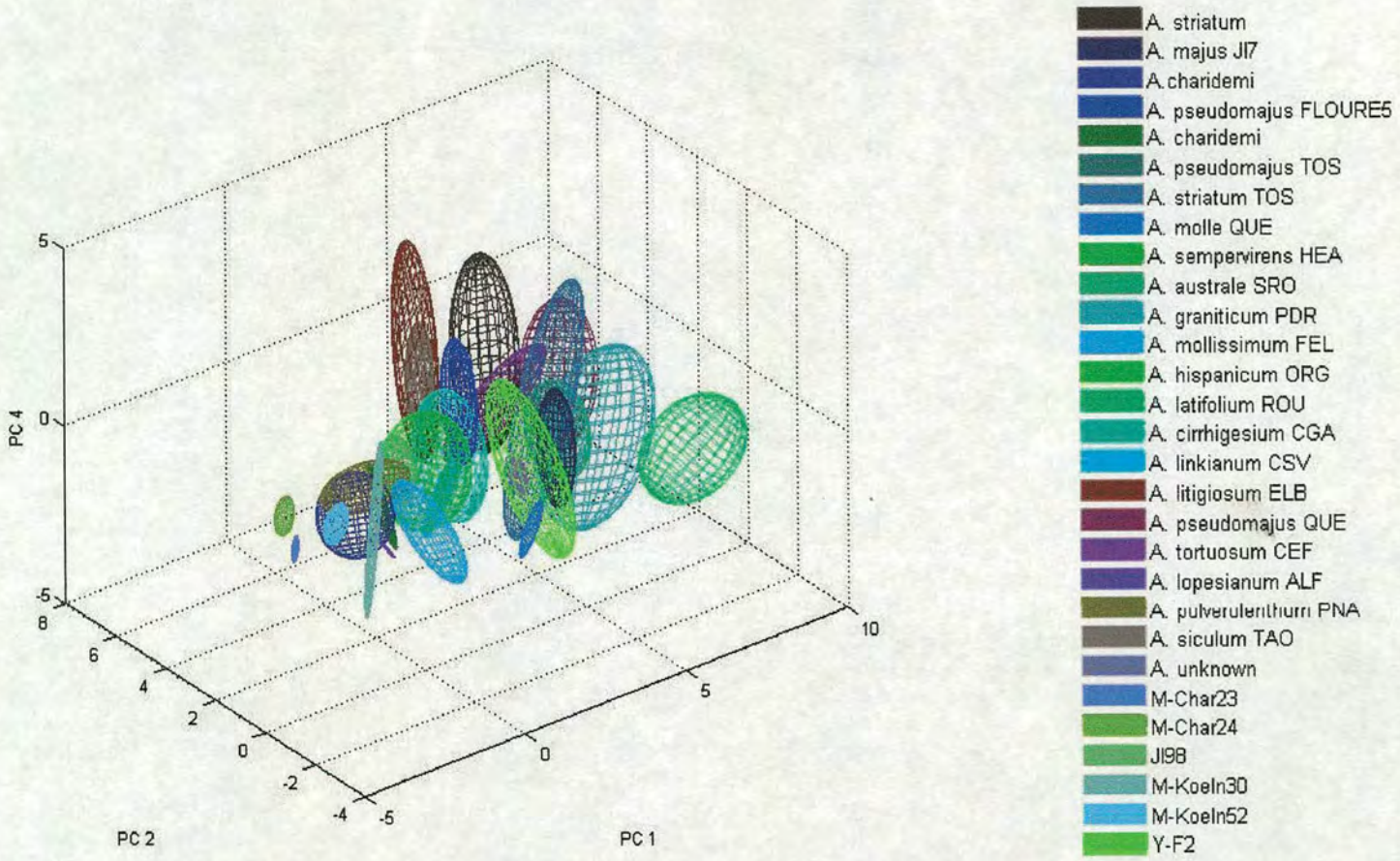



**Figure 3.2.3 Size and shape of leaves from 20 *Antirrhinum* species captured by the allometric model.** Representation of each species as a cloud in allometric space based on the $F_2$ between *A. majus* and *A. charidemi*. Each ellipsoid is based on leaf outlines from 2-14 individuals from each species.

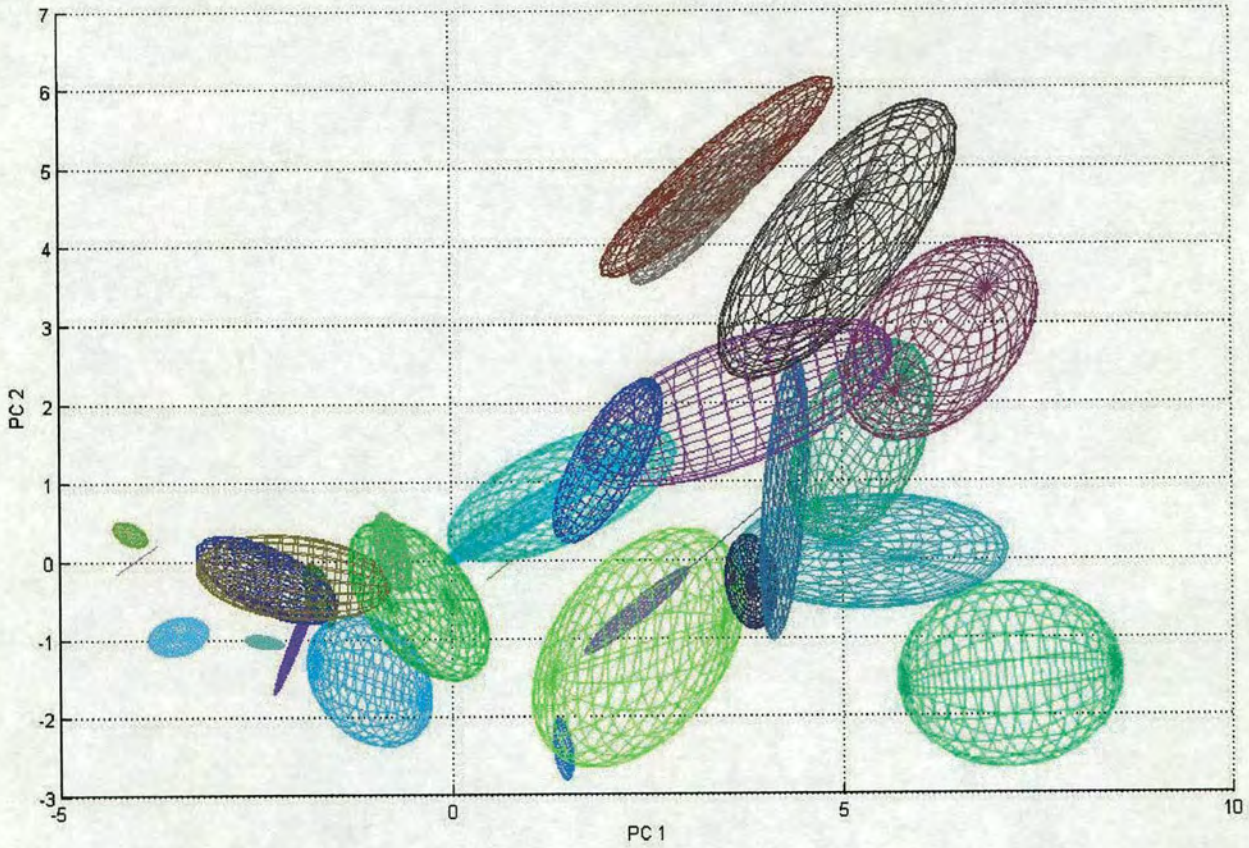



**Figure 3.2.3 (continued.)**

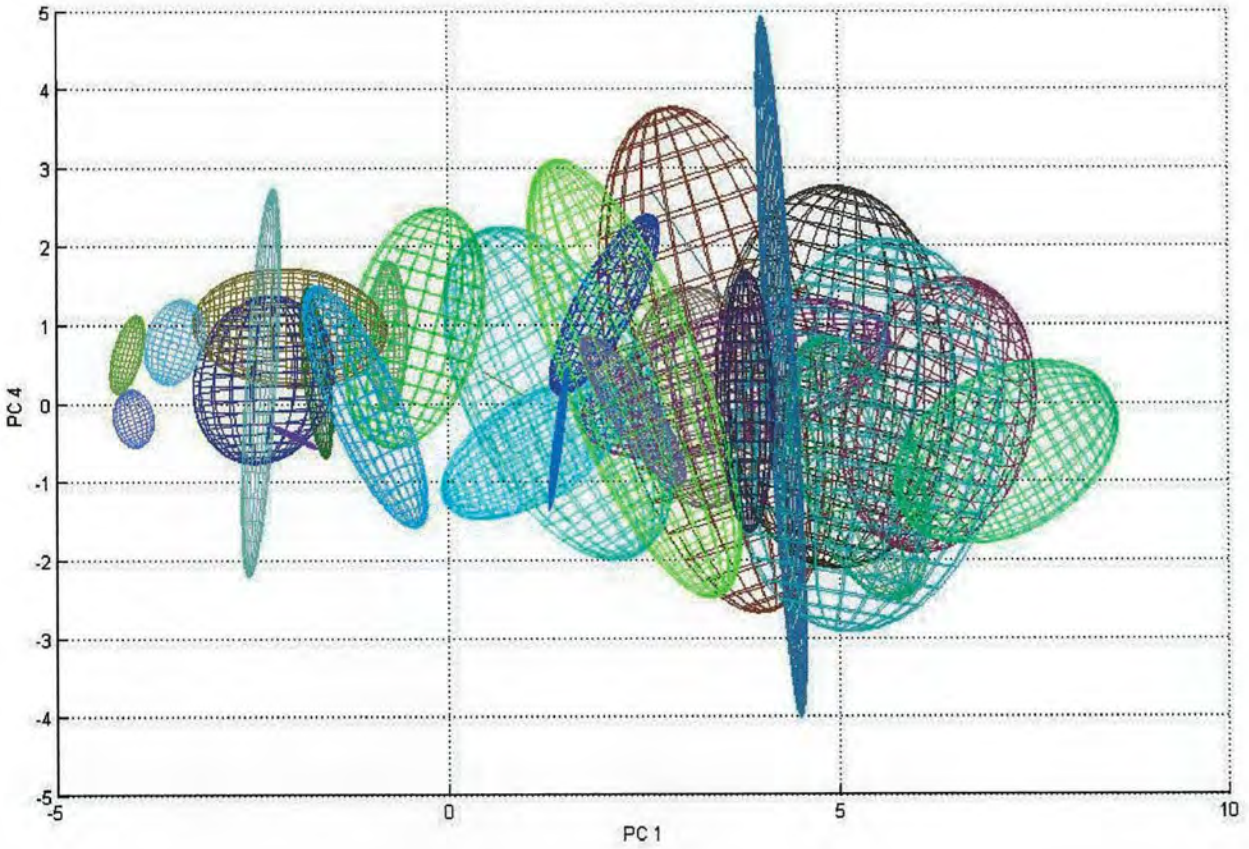



**Figure 3.2.3 (continued.)**

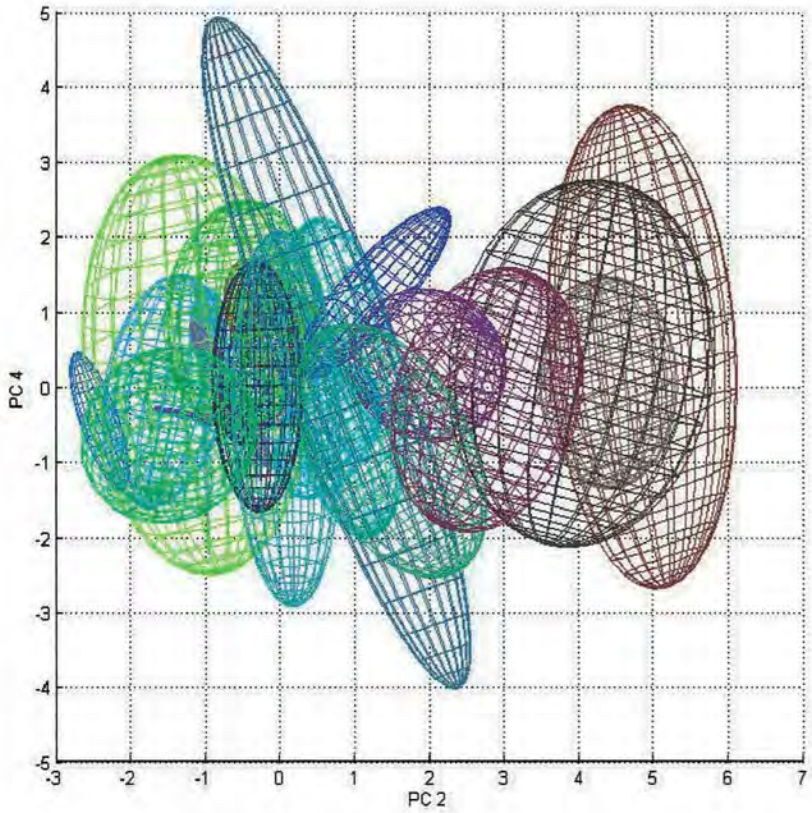



**Figure 3.2.3 (continued.)**

## GENOTYPIC APPROACHES

Analysis of traits in the F2 population of *A. majus* x *A. molle* identified traits that were positively or negatively correlated, suggesting that they might have a similar genetic basis. Attempts were therefore made to map the genes responsible for the differences in these traits between species as quantitative trait loci (QTL). Shape modelling had been successful in describing most of the combined variation in leaf shape and size within the population, allowing these traits to be used in QTL analysis to identify the genes involved.

QTL analysis involves identifying regions of the parental genomes responsible for phenotypic differences between offspring by regression of trait values onto genotypes. It therefore needs genotype data for the population and a genetic map to locate QTL within the genome.

## 3.3 Genotyping Amplified Fragment Length Polymorphisms (AFLPs)

### Identification and mapping of DNA polymorphisms

The first *Antirrhinum* genetic map had been made from the F2 of a cross between *A. majus* x *A. molle* and mainly built with Restriction Fragment Length Polymorphism (RFLP) and Cleaved Amplified Polymorphic Sequence (CAPS) markers (Schwarz-Sommer *et al.*, 2003); however, the mapping population did not have its phenotypes scored. The F2 population that I used for analysis of traits $n = 107$ was generated from a single $F_1$ progeny of the interspecific cross *A. majus* x *A. molle*, produced from the same parents as the original mapping population. A new genetic map ('Amanda-Yang map') was built for this population mainly with Amplified Fragment Length Polymorphisms (AFLPs). Being PCR based, AFLP requires no prior sequence knowledge and detects many more genetic loci than RFLP, but has the disadvantage that markers are usually treated as dominant. Some CAPS primer combinations from the old map were also used in building the new map; this also gave the opportunity to compare the old and new maps and to co-align them. This map is described in more detail below.

A further approach was to integrate data from Zsuzsanna Schwarz-Sommer's population with mine to build a combined map from a larger number of individuals. I therefore used the same AFLP primer combinations used in the Amanda-Yang map to genotype Zsuzsanna's F2 population. As a first step, I built a combined map from the German F2 population that incorporated AFLP and Zsuzsanna's previous RFLP data.

Having demonstrated that this was possible, a final approach was to put the combined genotype data for Zsuzsanna's population together with the genotype data for the Amanda-Yang population to build a new combined map ($n$ = 203). The Amanda-Yang F2 mapping population was produced from a different F1 plant to Zsuzsanna's population, nevertheless, both F1 plants were from the same parents. The *A. majus* parent, 165E or JI98, was an inbred line and should therefore have contributed the same alleles to the two mapping populations. Although the *A. molle* parent was an outbreeding wild accession that was heterozygous at many loci, there was a 50% chance that the same allele of a heterozygous locus had been inherited by the two mapping populations.

F3 populations from both F2 mapping populations were then grown under the same conditions and scored for a limited number of phenotypes. Each population was analysed for QTLs separately with its own F2 map and genotype data and then both F3 populations were used together in QTL analysis with the combined map to compare the detection, position and effects of QTLs.

**Table 3.3 Difference between Restriction Fragment Length Polymorphisms (RFLPs) and Amplified Fragment Length Polymorphisms (AFLPs).**

| RFLPs | AFLPs |
|---|---|
| • Developed early eighties | • Late eighties to early nineties |
| • Hybridization based | • Polymerase chain reaction (PCR) based |
| • Requires use of a library of DNA fragment cloned into vectors | • Requires no prior sequence knowledge |
| • Laborious and only one to a few loci are detected per assay | • Combines the advantage of the time efficiency from PCR-based markers and the reliability of RFLP markers |
| • Detects polymorphisms in known sequences | • Polymorphic sequences not known |
| • Alleles often co-dominant | • Alleles most easily analysed as dominant markers |

## Building the Maps

### 1. The Edinburgh F2

A new F2 linkage map was built with the information from six different AFLP primer combinations that generated 164 polymorphic fragments (Table 3.3.1), and had been scored conservatively as co-dominant markers on the basis of fluorescent signal intensity. Where the signal intensity was intermediate between that expected of a homozygote and a heterozygote, the genotype was scored as for a dominant locus. Another 30 loci were detected as codominant CAPS alleles. Genotype analysis had been carried out by Amanda Borking and Thomas Gübitz.

I analysed a total of 194 markers for linkage with JoinMap® V3.0 (Van Ooijen and Voorrips, 2001). In the first step, linkage groups were calculated based on the logarithm of the odds (LOD) ratio for each possible marker pair. The LOD value expresses the likelihood of linkage by comparing the probabilities of random association of markers in the F2 population to association caused by linkage. In this population, the LOD values used were in the range of 5-8, which is considered relatively stringent. In the second step, a linear order of markers within a linkage group was calculated with LOD value of >1.0 and the Kosambi mapping function. In twenty-two cases, either AFLP primer combinations revealed pairs of markers in repulsion with recombination fractions (RFs) of zero, representing potentially codominant alleles of the same locus (see Figure 3.3), or markers of the same primer combination were linked in coupling with RF = 0, therefore representing potentially identical loci. These markers are represented in Figure 3.3 as single loci. The minimum estimate for the number of mapped loci was therefore 142. Hence, eventually 142 markers were positioned on the map in eight linkage groups with a total distance of 494 cM (average interval of 2.5 cM; Figure 3.3). The eight linkage groups could be assigned to the eight chromosomes of a classical genetic map (Stubbe, 1966) by the presence of common markers. Therefore the eight linkage groups could be named as in the classical genetic map. The characteristics of this map are presented in Table 3.3.2.

**Table 3.3.1 Six different AFLP primer combinations and the number of codominant polymorphic fragments found for each primer combinations.**

| *Mse* I primers \ *EcoR* I primers | P11-GAA | P12-GAC | P14-GAT |
|---|---|---|---|
| CAC | 26 | | |
| AGA | 21 | | |
| AGG | 33 | | |
| AGC | | 13 | 25 |
| ACA | | 26 | |
| CAT | 17 | | 3 |
| | | TOTAL | 164 |

**Table 3.3.2 Characteristics of the Amanda-Yang linkage map**

| Linkage group | Length (cM) | No. of codominant markers | No. of dominant markers | Total no. of markers | No. of loci |
|---|---|---|---|---|---|
| 1 | 72 | 21 | 3 | 24 | 19 |
| 2 | 46 | 26 | 5 | 31 | 17 |
| 3 | 75 | 22 | 4 | 26 | 21 |
| 4 | 48 | 25 | 3 | 28 | 23 |
| 5 | 33 | 14 | 2 | 16 | 11 |
| 6 | 46 | 15 | 3 | 18 | 11 |
| 7 | 58 | 19 | 6 | 25 | 18 |
| 8 | 116 | 22 | 4 | 26 | 22 |
| Total | 494 | 164 | 30 | 194 | 142 |
| Mean | 61.75 | 20.50 | 3.75 | 24.25 | 17.75 |

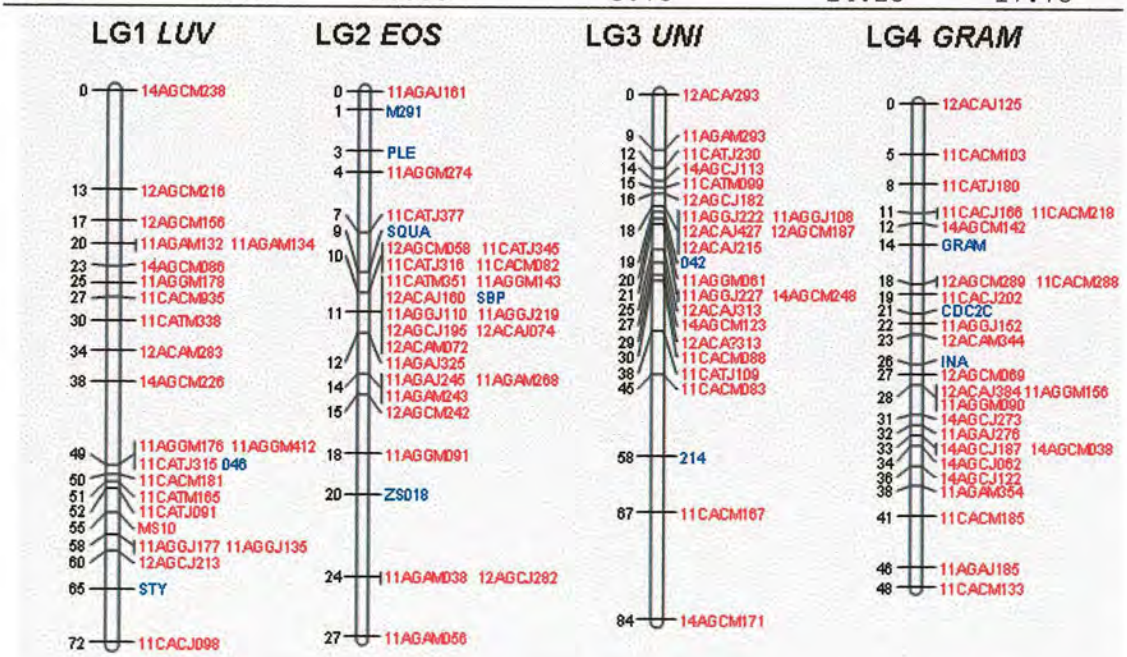



**Figure 3.3 Linkage maps of Antirrhinum derived from an interspecific hybrid between *A. majus* and *A. molle*.** Distances are given in centimorgans (Kosambi). Loci with codominant AFLP markers are in red. Loci with dominant CAPS markers are in blue.

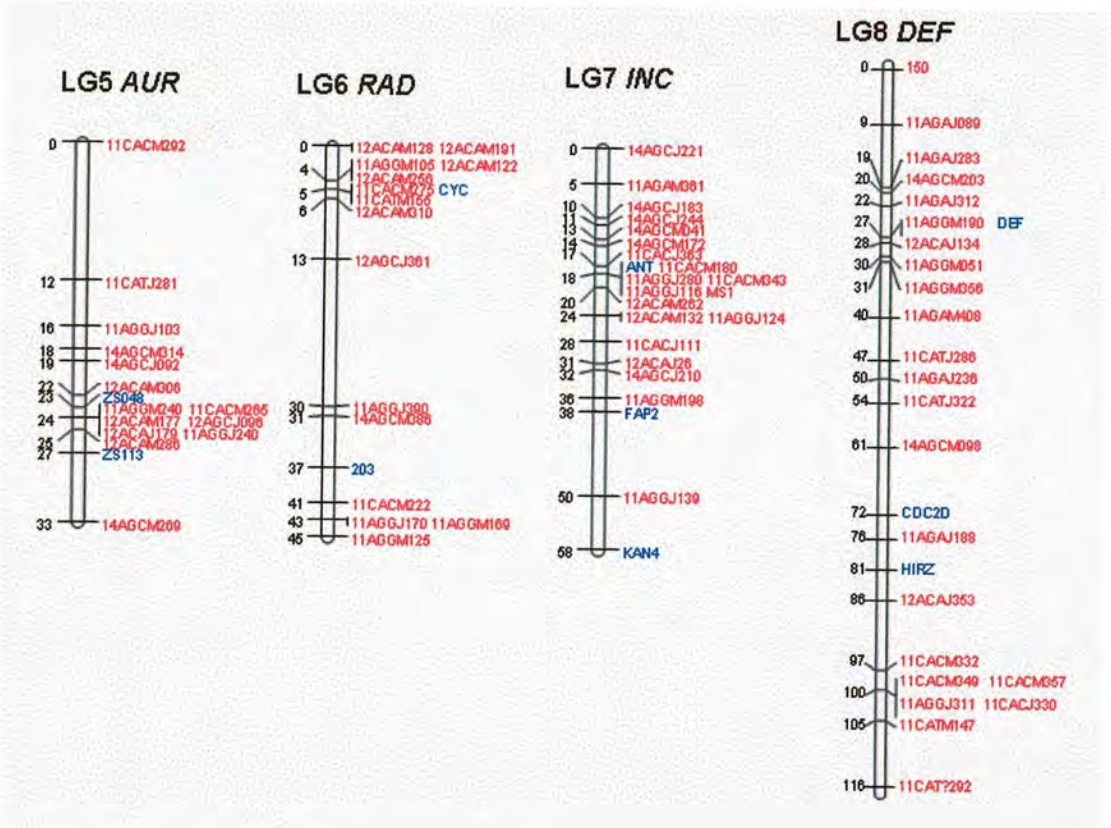



**Figure 3.3 (Continued).**

## 2. German F2 (RFLP) map

Previously, a linkage map for Zsuzsanna's F2 population had been built with Enrique Ritter's method (Ritter and Salamini, 1996) using mainly RFLP markers. In order to obtain a map comparable to the Amanda-Yang map, Zsuzsanna's genotype data were reanalysed with JoinMap® V3.0 using the same condition used for the Amanda-Yang map (Figure 3.3.1). The new map for Zsuzsanna's data resolved eight linkage groups with a total length of 453 cM (average = 56.6 cM). The reanalysed map was aligned with the Amanda-Yang map on the basis of common CAPS makers. The total length of Zsuzsanna's map is 41 cM shorter than the Amanda-Yang map with average 3.8 cM difference in each linkage group, although the Amanda-Yang map contained 70 fewer markers than Zsuzsanna's map. One explanation for the difference in map length is that Zsuzsanna's map contained a high proportion of coding loci, which were found to cluster in the middle of chromosome arms, while the Amanda-Yang map contained more AFLPs which might represent more non-coding regions and therefore map more of the genome. Alternatively, errors in scoring AFLP loci as co-dominant loci might have

contributed to the increased length of the Amanda-Yang map because each mis-scoring increases the estimated distance to neighbouring loci. The differences in both maps are presented in Table 3.3.3. The comparisons of both maps are shown in Figure 3.3.1.

**Table 3.3.3 Characteristics of the Zsuzsanna's RFLP linkage map**

| | **Amanda-Yang's AFLP map** | | **Zsuzsanna's RFLP map** | |
|---|---|---|---|---|
| Linkage group | Length (cM) | No. of markers | Length (cM) | No. of markers |
| 1 | 72 | 24 | 63 | 40 |
| 2 | 46 | 31 | 25 | 32 |
| 3 | 75 | 26 | 79 | 32 |
| 4 | 48 | 28 | 53 | 47 |
| 5 | 33 | 16 | 64 | 26 |
| 6 | 46 | 18 | 35 | 19 |
| 7 | 58 | 25 | 65 | 30 |
| 8 | 116 | 26 | 69 | 38 |
| Total | 483 | 194 | 453 | 264 |
| Mean | 60.4 | 24.3 | 56.6 | 33.0 |
| average interval | 2.5 cM | | 1.7 cM | |

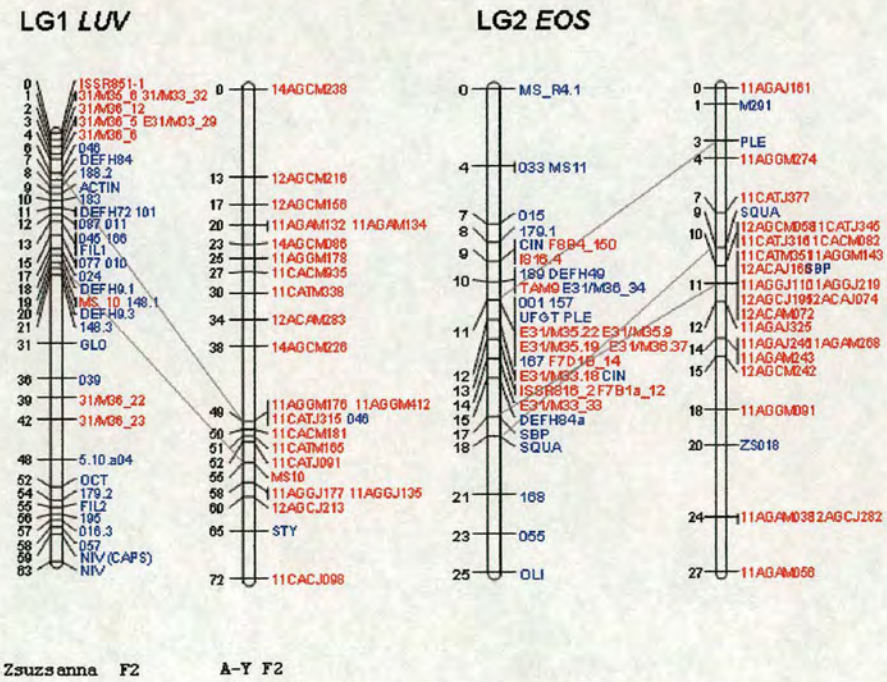



**Figure 3.3.1 Linkage maps of Antirrhinum derived from an interspecific hybrid between *A. majus* and *A. molle*.** Distances are given in centimorgans (Kosambi). AFLP markers are in red and CAPS markers in blue. Grey lines connect loci that were mapped in both populations (as CAPS or RFLPs).

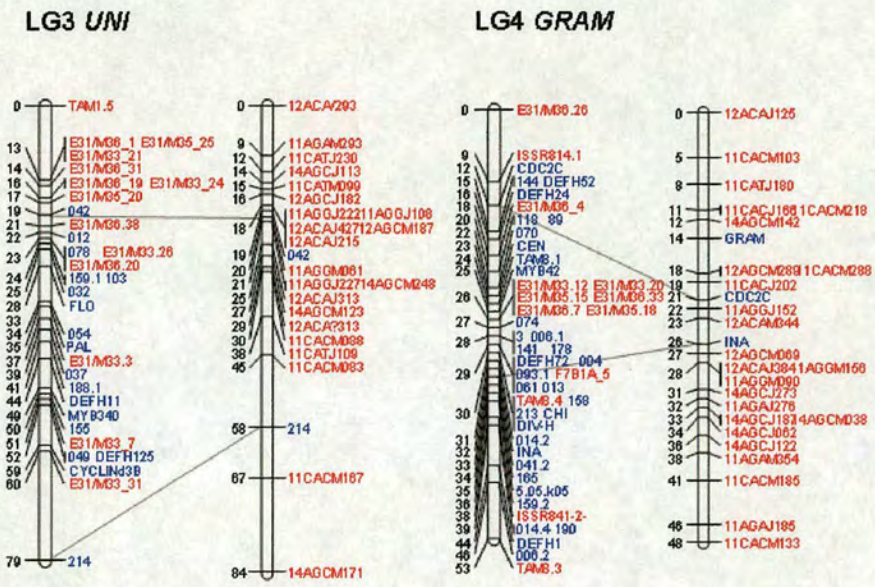



Figure 3.3.1 (Continued).

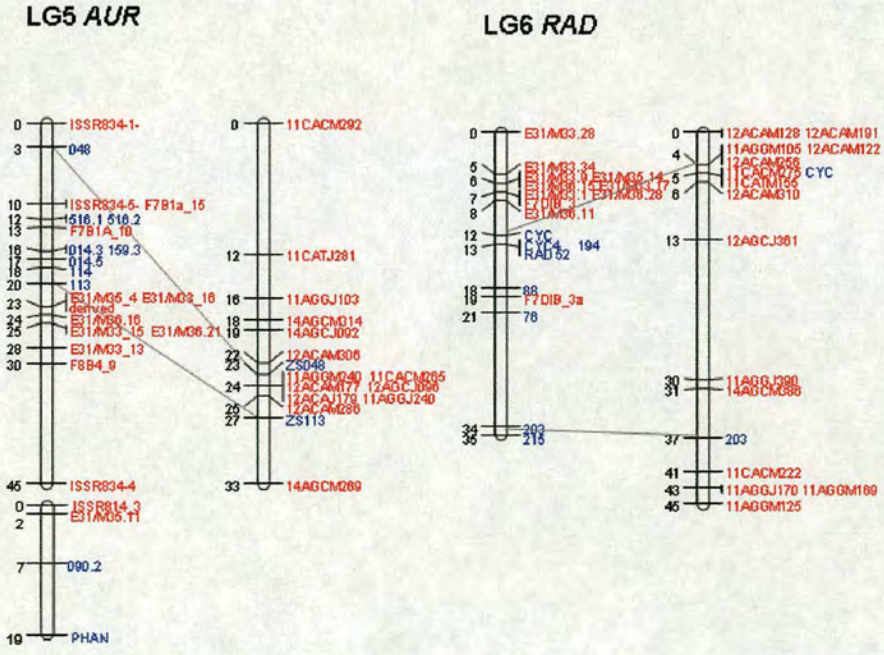



Figure 3.3.1 (Continued).

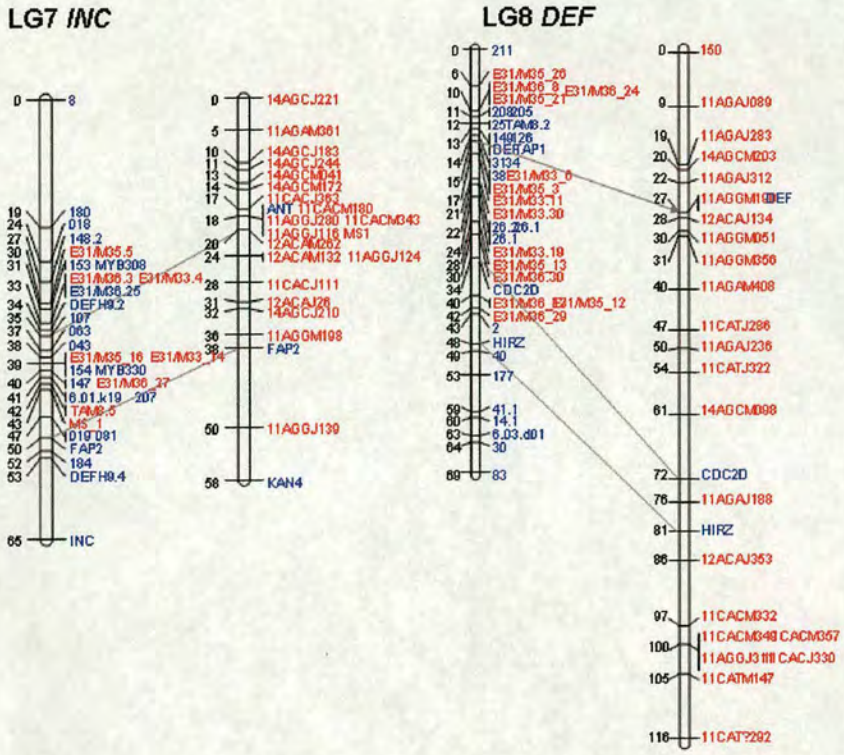



**Figure 3.3.1 (Continued).**

## 3. German F2 (AFLP) map and combined map

To analyse QTLs in two different F3 populations, a comparable map was needed. The original Zsuzsanna map was build with mainly RFLP markers while the Amanda-Yang map consisted mainly of AFLPs. Although common loci had been mapped in both populations as RFLPs or CAPS, the two maps could not be co-aligned accurately (see Figure 3.3.1). To be able to analyse and compare the QTL results from the two populations, the German F2 population was genotyped for the same AFLP primer combinations used in the Amanda-Yang F2 mapping population. A total of 177 AFLP loci were scored as co-dominant markers where possible and used to construct a map, using only the AFLP data for the German population. The resulting German F2 AFLP map showed seven linkage groups with total length of 221 cM (average 31.6 cM), but consisted of only 34 markers (20% of the total) mapped at 34 loci with an average interval of 4.9 cM. The characteristics of this map are presented in Table 3.3.4. The inability to recover eight linkage groups using only AFLP markers was not unexpected. For example, linkage group 6 contains the self-incompatibility *S* locus which is functional in *A. molle*

and prevents formation of F2 plants homozygous for the *A. molle S* allele. This makes it difficult to recover linkage group 6 in maps containing a high proportion of dominant alleles from *A. majus* because these cannot distinguish plants homozygous for a region around the *S* locus of *A. majus* from the heterozygotes. Because no *A. molle* homozygotes are recovered, there is not sufficient information to map this region. Similarly, incorporation of markers that can be scored unambiguously as co-dominant (e.g. CAPS or RFLPs) usually increases the proportion of dominant markers (e.g. AFLPs) that can be mapped. However, the low proportion of AFLPs that could be mapped in this case was exceptionally low.

**Table 3.3.4 German F2 AFLP map**

| Linkage group | length (cM) | no of loci | no of markers |
|---|---|---|---|
| 1 | 28 | 8 | 8 |
| 2 | 36 | 4 | 4 |
| 3 | 19 | 3 | 3 |
| 4 | 20 | 3 | 3 |
| 5 | 29 | 2 | 2 |
| 6 | 64 | 11 | 11 |
| 7 | 25 | 3 | 3 |
| total | 221 | 34 | 34 |
| mean | 31.6 | 4.9 | 4.9 |

The markers used for this AFLP map were therefore put together with the genotype data for the RFLP and CAPS markers previously used to build Zsuzsanna's map to build a combined map for the German F2 population. The combined map was analysed by using JoinMap V3.0 under the same settings as for the Amanda-Yang map. The combined map resolved 10 linkage groups containing a total of 224 markers. Finding markers that had previously mapped to the same linkage group in different linkage groups in this map, showed that mapping had not resolved chromosomes 5 and 8 into single linkage groups. However mapping 224 markers supported the view that the number of loci recovered in the map that used only AFLP markers had been caused by low information content of the AFLP markers. Eliminating markers that mapped to the same loci, 168 loci were mapped with average intervals of 3.2 cM (Figure 3.3.2). The total length of 544 cM (average 54.4 cM per linkage group) was significantly larger than the map made with RFLP markers alone (453 cM). Because the RFLP map was estimated to contain ~93% of the genome (Schwarz-Sommer et al., 2003), the increase in map length is unlikely to result from mapping of new regions of the genome. Instead, it is more likely to result from mis-scoring of marker genotypes, because each mis-scoring introduces at least one recombination event into the

mapping calculations. The characteristics of this map are presented in Table 3.3.5.

**Table 3.3.5 New German F2 Combined Map**

| Linkage group | length (cM) | no of loci | no of markers |
|---|---|---|---|
| 1 | 57 | 10 | 10 |
| 2 | 61 | 22 | 33 |
| 3 | 93 | 27 | 35 |
| 4 | 66 | 30 | 42 |
| 5 | 76 | 19 | 24 |
| 5-1 | 19 | 4 | 4 |
| 6 | 30 | 13 | 20 |
| 7 | 69 | 10 | 15 |
| 8 | 24 | 18 | 25 |
| 8-1 | 49 | 15 | 16 |
| total | 544 | 168 | 224 |
| mean | 54.4 | 16.8 | 22.4 |

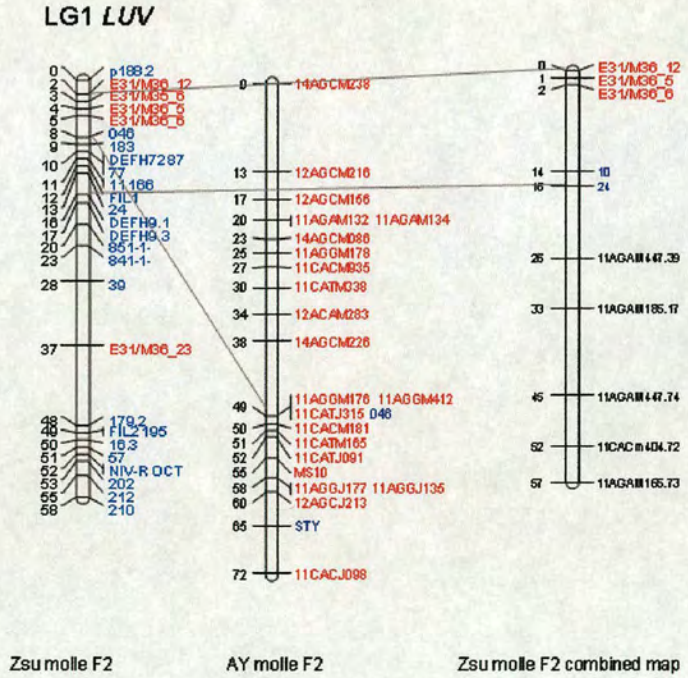



**Figure 3.3.2 Combined linkage groups of the German F2 population.** Distances are given in centimorgans (Kosambi). Loci with co-dominant AFLP markers are in regular black and red colour. Loci with dominant CAPS markers are in blue colour.

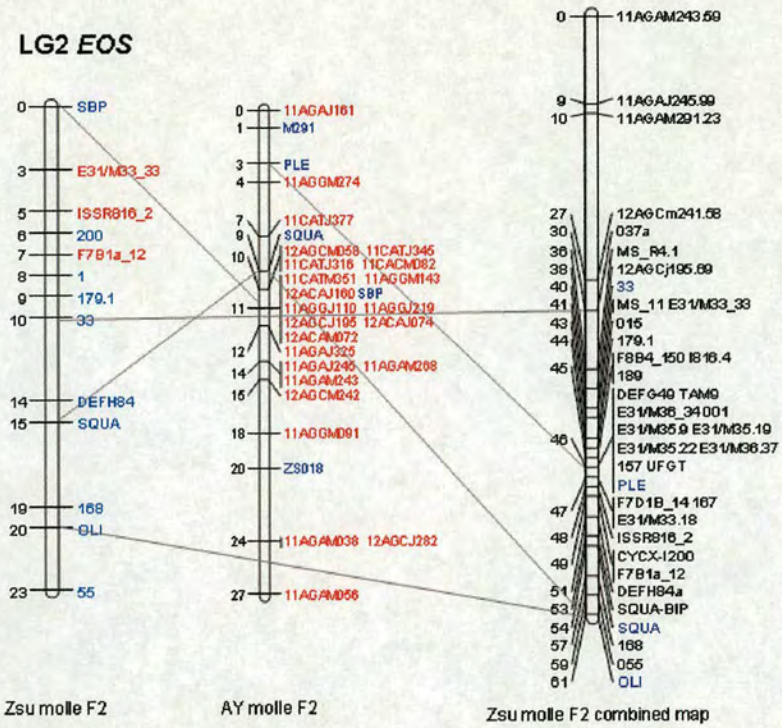



**Figure 3.3.2 (Continued).**

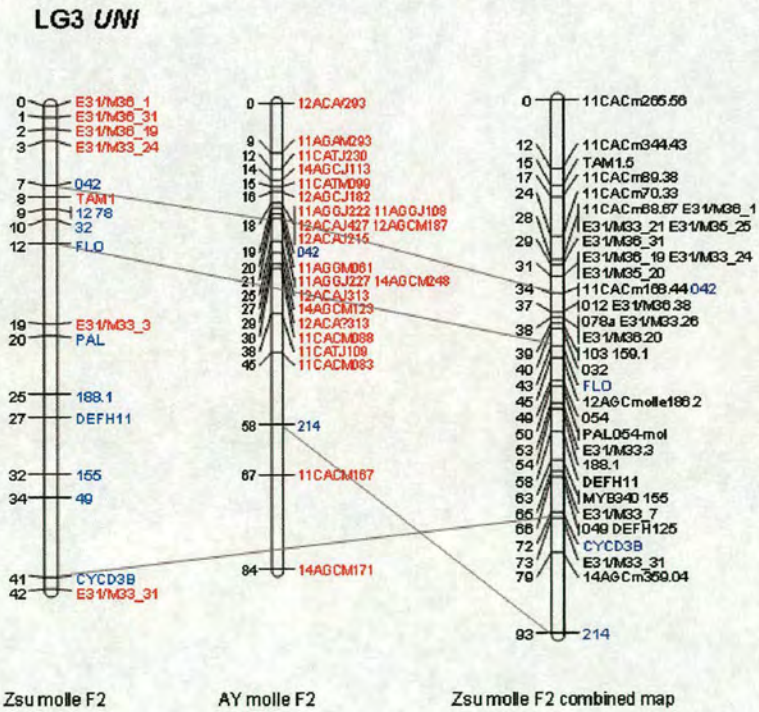



**Figure 3.3.2 (Continued).**

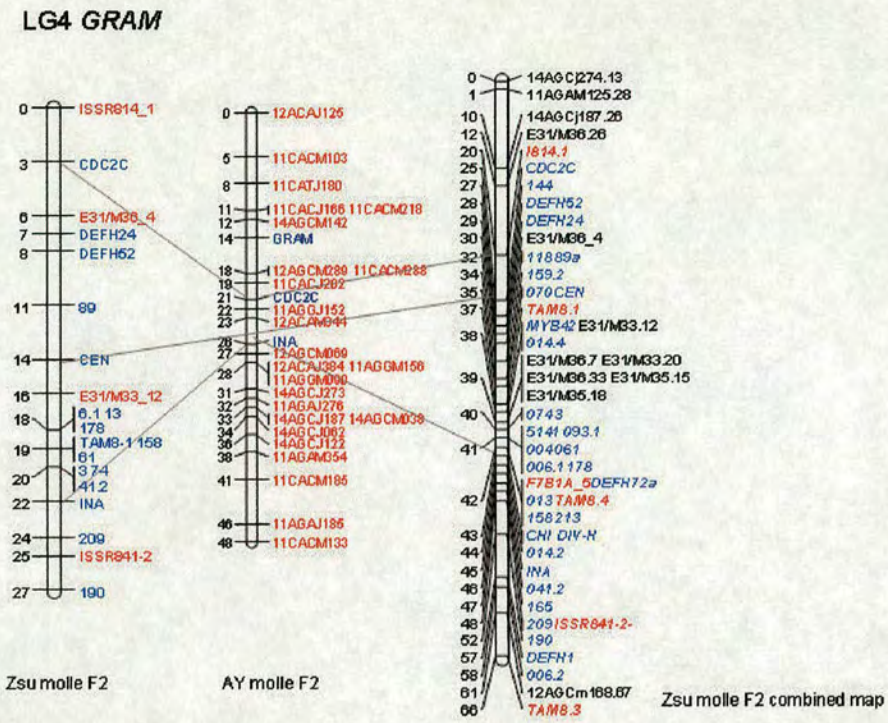



**Figure 3.3.2 (Continued).**

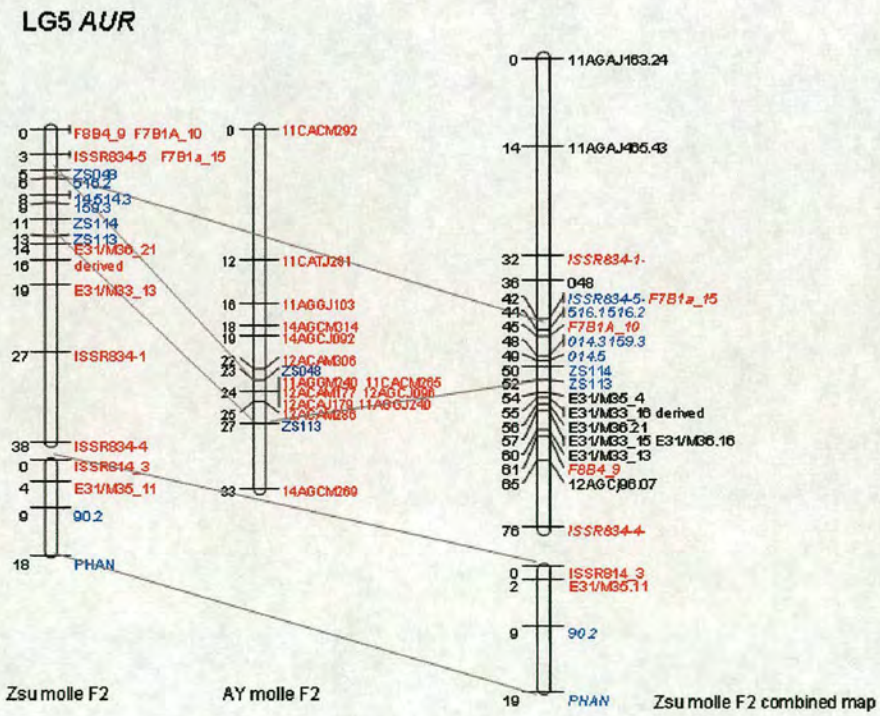
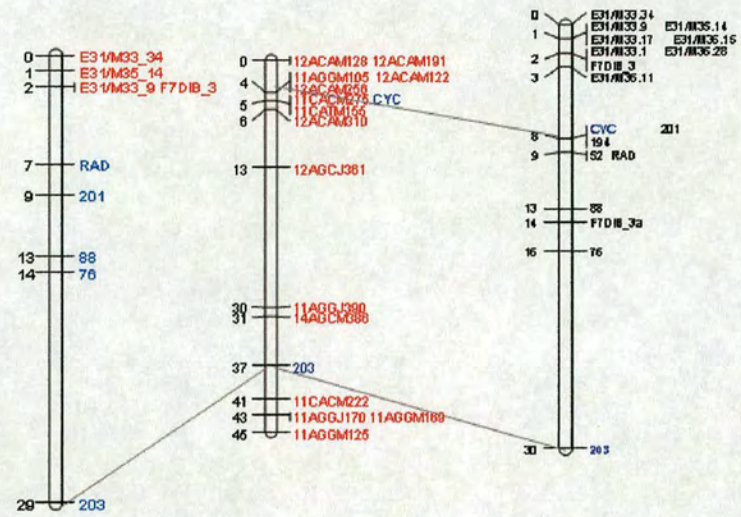



**Figure 3.3.2 (Continued).**

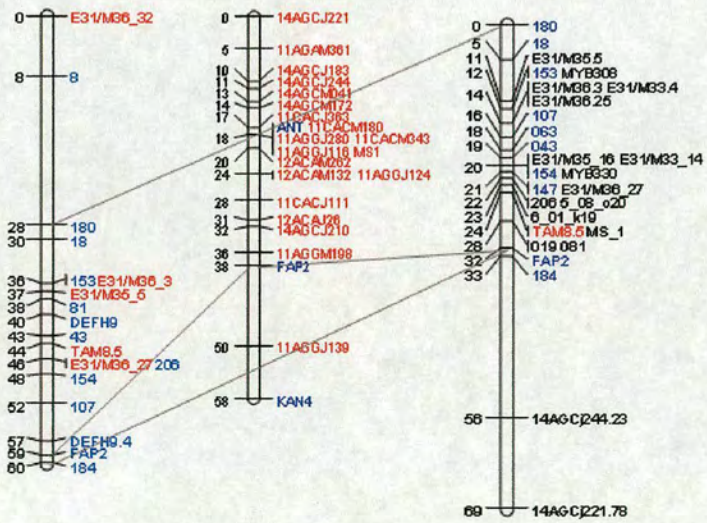
LG6 *RAD*




Zsu molle F2 AY molle F2 Zsu molle F2 combined map

**Figure 3.3.2 (Continued).**

LG7 *INC*




Zsu molle F2 AY molle F2 Zsu molle F2 combined map

**Figure 3.3.2 (Continued).**

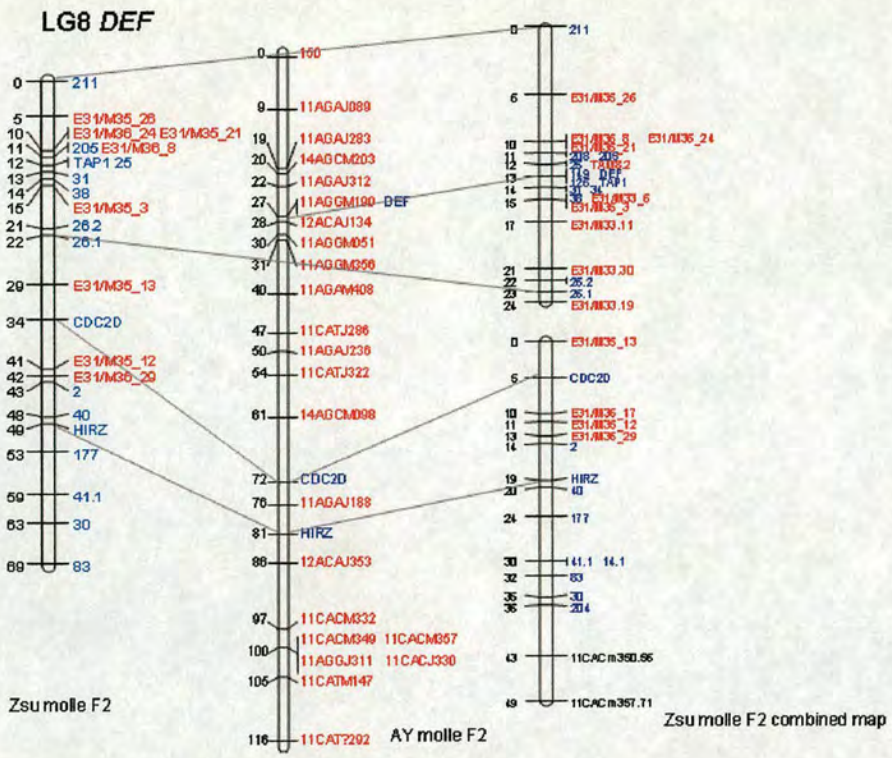



**Figure 3.3.2 (Continued).**

## 4. German-Edinburgh combined map

Having genotyped Zsuzsanna's F2 population for the same AFLP primer combinations used with the Amanda-Yang population and demonstrated that it was possible to integrate the AFLP and RFLP data to build a single map, attempts were made to produce a single map from the combined genotype data of the two populations. To do this, the German F2 individuals were relabelled as 1 to 96 and the Edinburgh F2 population was relabelled as 97 to 203. The combined population therefore consisted of 203 different individuals. For RFLP and some AFLP markers absent from one population, genotype data were recorded as missing for part of the combined population. A map was constructed in Joinmap® V3.0, as before. The combined map showed 12 linkage groups with a total length of 415 cM (average 34.6 cM). Three hundred and seventy-seven markers were mapped to 215 loci with an average interval of 2.0 cM between loci (Figure 3.3.3). In comparison to the previous Zsuzsanna map, linkage group 1 was split into two separate linkage groups with a total length of 54 cM compared to 63 cM in the Zsuzsanna map. Linkage group 2 was 27 cM, similar to the 25 cM in the Zsuzsanna

map, linkage group 3 was 64 cM compared with 79 cM, and linkage group 4 was 60 cM compared to 53 cM. Linkage group 5 of the Zsuzsanna map was represented by two separate groups in the combined map with a total length of 60 cM, 4 cM shorter than linkage group 5 in the Zsuzsanna map. Similarly, linkage group 6 was split into two groups with a total length of 31 cM compared to 35 cM in the previous map and linkage group7 was represented by two groups with a shorter total length of 45 cM compared 69 cM. Finally, linkage group 8 was also split into two groups with a total length of 83 cM compared to 69 cM in the previous map. In total, about 76.5% of the loci were mapped in the Germany-Edinburgh combined map. The characteristics of this map are presented in Table 3.3.6.

**Table 3.3.6 Germany-Edinburgh combined map**

| Linkage group | length | no of loci | no of markers |
|---|---|---|---|
| 1 | 48 | 27 | 46 |
| 1-1 | 6 | 6 | 8 |
| 2 | 27 | 21 | 51 |
| 3 | 64 | 24 | 37 |
| 4 | 60 | 36 | 86 |
| 5 | 40 | 19 | 30 |
| 5-1 | 20 | 4 | 4 |
| 6 | 9 | 7 | 9 |
| 6-1 | 22 | 11 | 16 |
| 7 | 39 | 19 | 23 |
| 7-1 | 6 | 5 | 9 |
| 8 | 37 | 19 | 31 |
| 8-1 | 46 | 17 | 27 |
| total | 424 | 215 | 377 |
| mean | 32.6 | 17.3 | 30.7 |

LG1 *LUV*

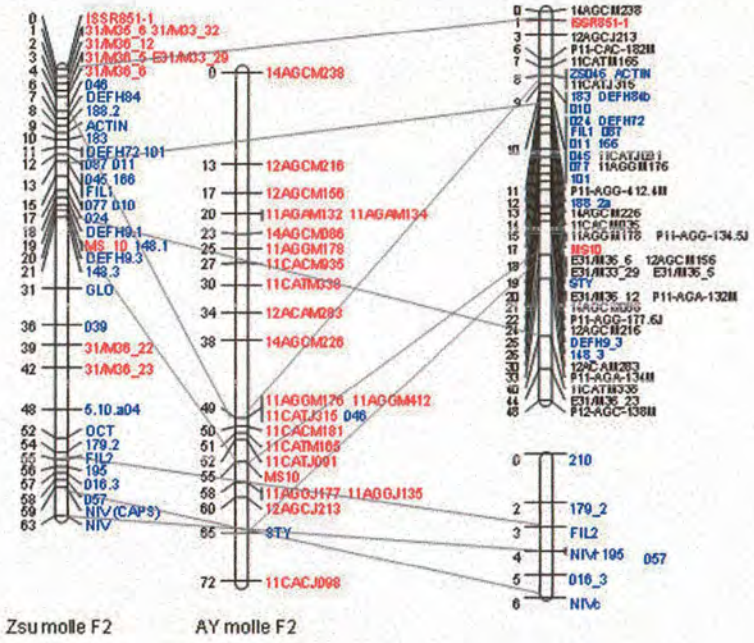



**Figure 3.3.3 Linkage groups of both Germany and Edinburgh F2 *Antirrhinum* derived from an interspecific hybrid between *A. majus* and *A. molle*.** Distances are given in centimorgans (Kosambi).

LG2 *EOS*

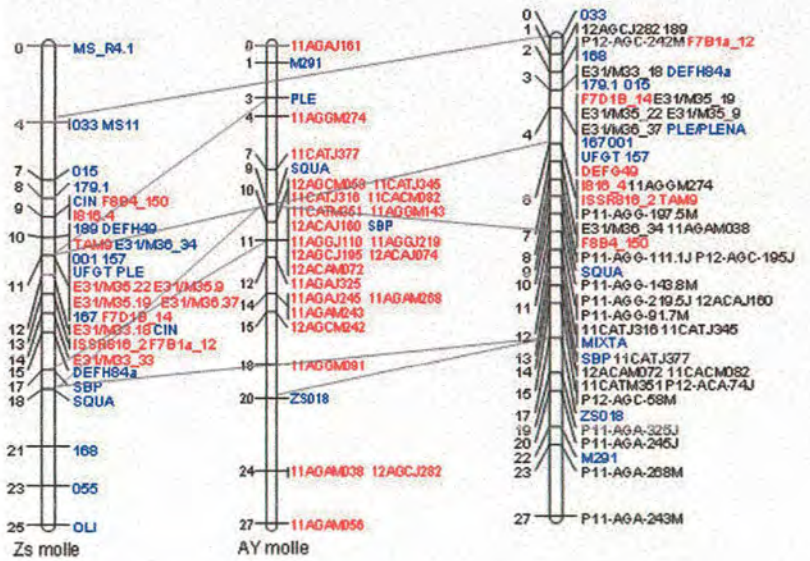



**Figure 3.3.3 (Continued).**

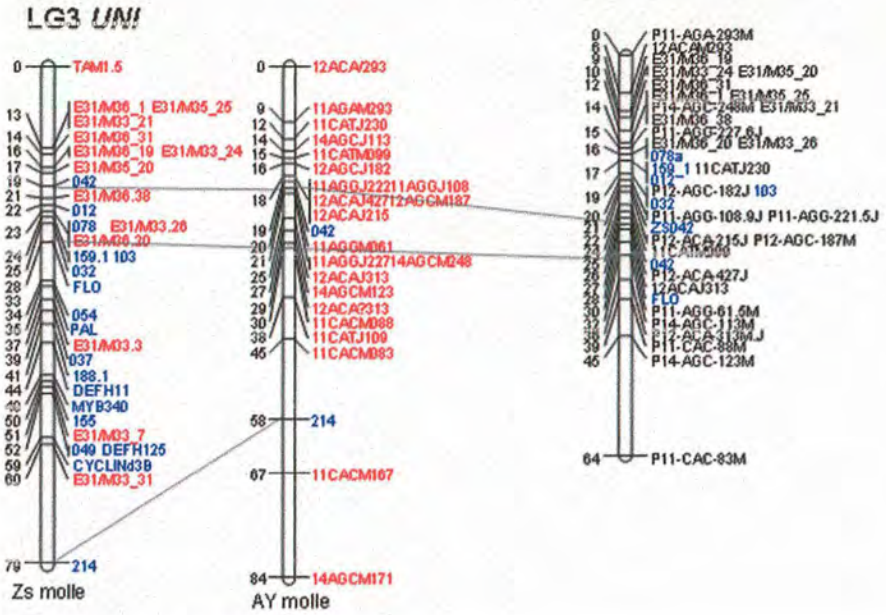



**Figure 3.3.3 (Continued) .**

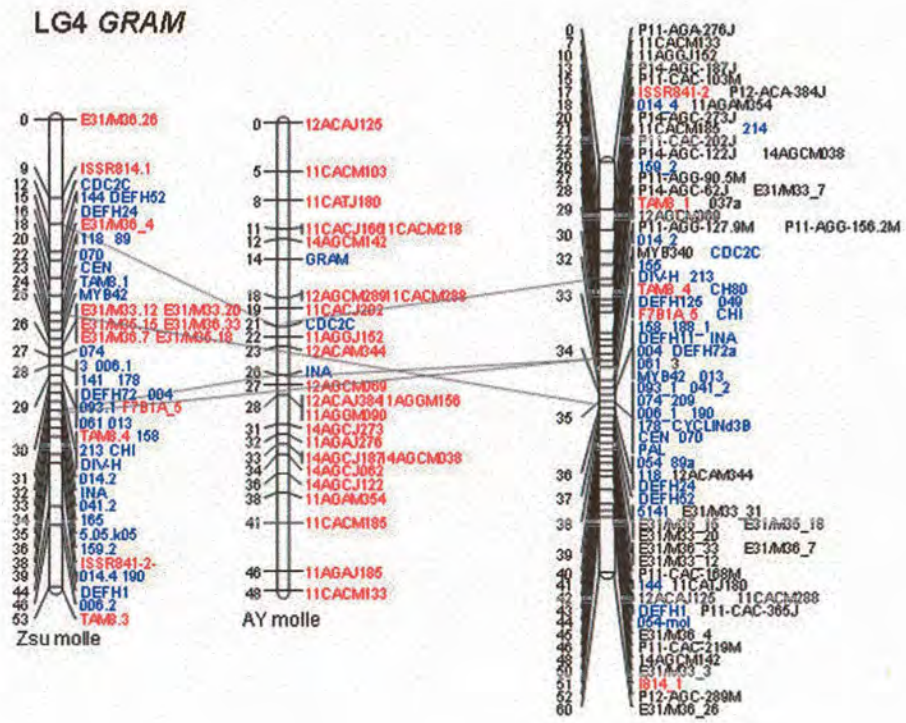



**Figure 3.3.3 (Continued).**

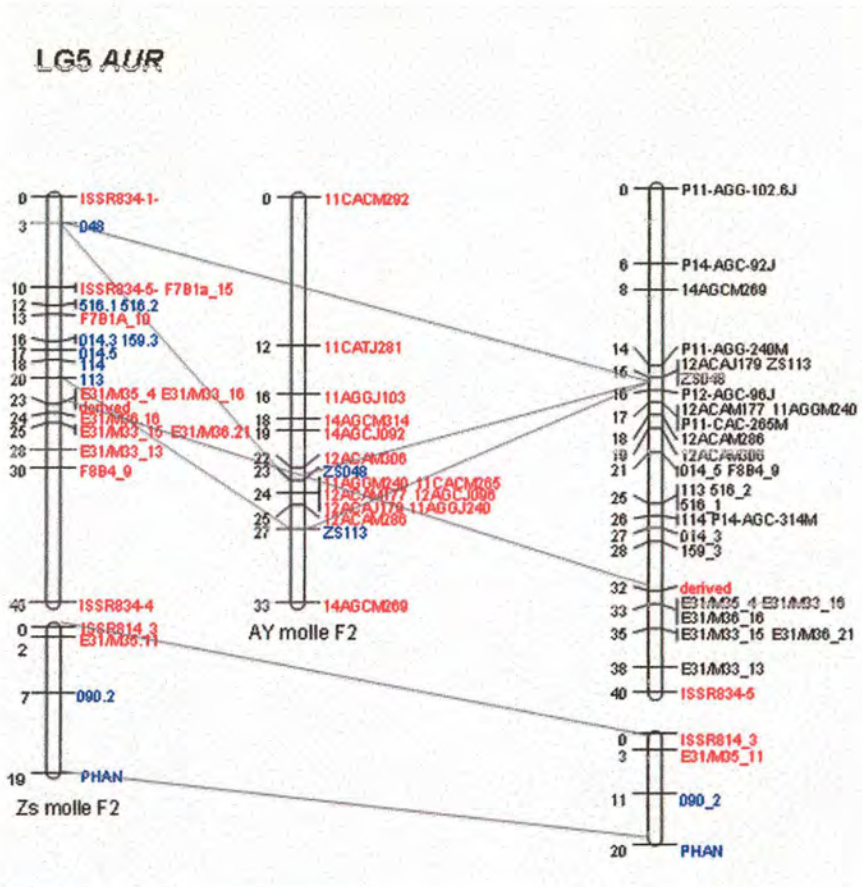



Figure 3.3.3 (Continued) .

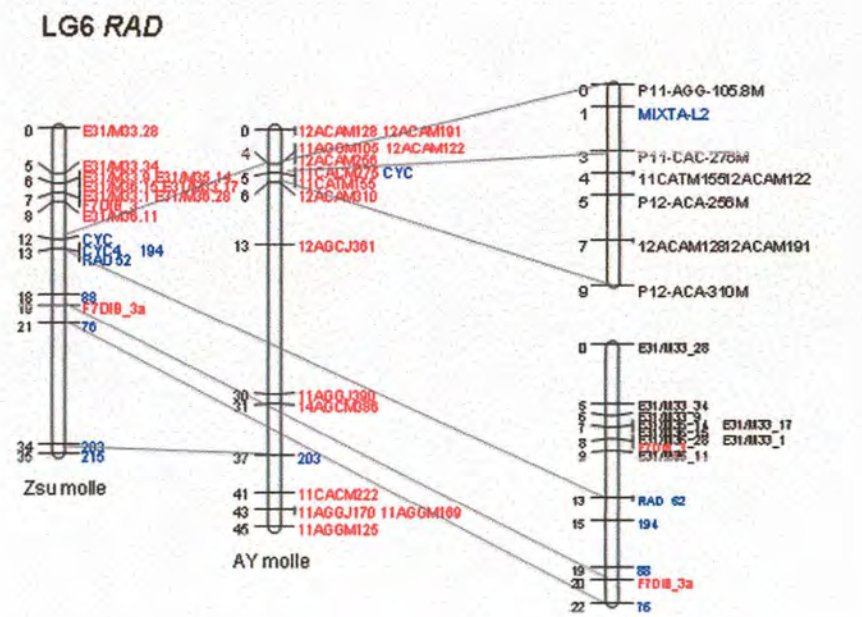



Figure 3.3.3 (Continued).

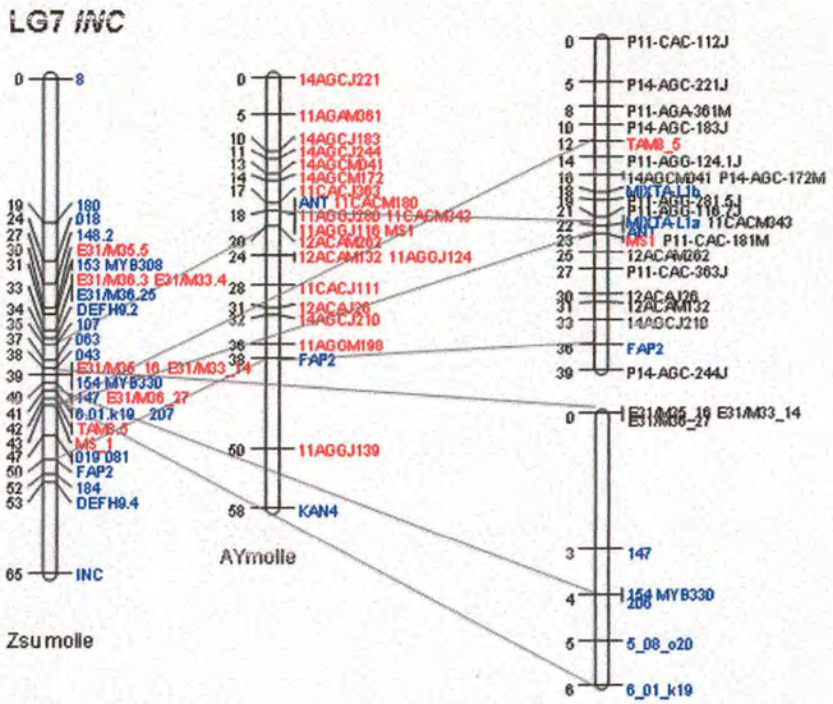



Figure 3.3.3 (Continued)

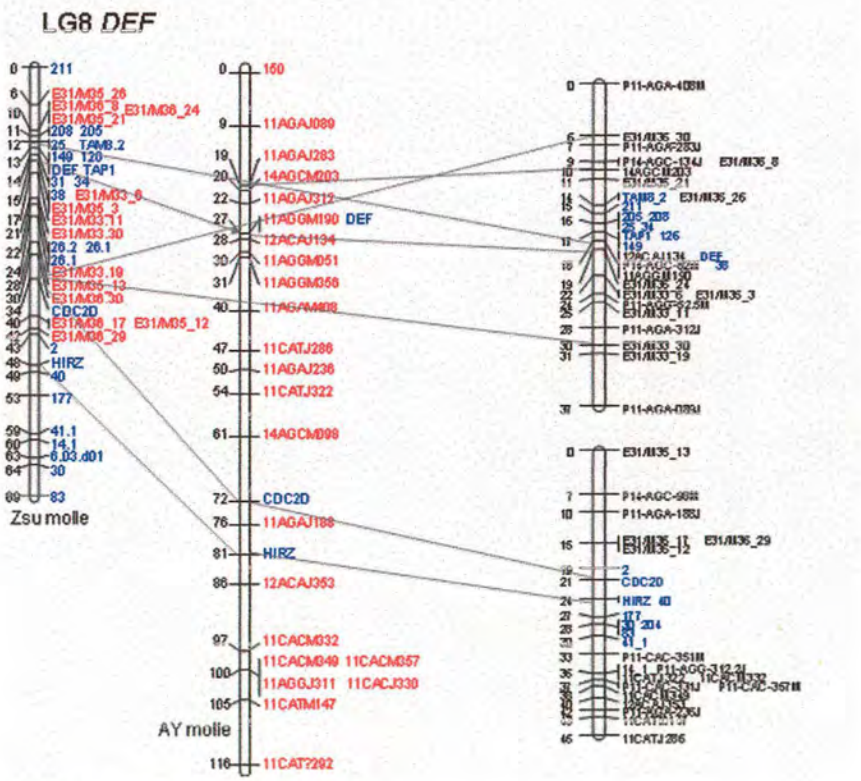



Figure 3.3.3 (Continued) .

## Summary of results

Five different linkage maps were made from mainly AFLP, RFLP and some CAPS markers. In the Amanda-Yang map, made with mainly AFLP and CAPS markers, eight linkage groups were identified with a total length of 483 cM. These contained 194 markers mapped to 142 different loci (average interval = 3.4 cM). The markers in this map were mainly AFLP and CAPS. AFLP and CAPS markers were scored as codominant.

Zsuzsanna's RFLP map was re-built with mainly RFLP and CAPS. A total of 264 markers in 179 different loci were mapped to 8 different linkage groups with a total length of 453 cM (average interval = 2.5 cM). The markers in this map were mainly RFLP and CAPS where both RFLP and CAPS were scored as co-dominant markers where possible.

An AFLP map for Zsuzsanna's population resolved only seven linkage groups and consisted only of 34 markers. However, an AFLP-RFLP combined map for Zsuzsanna's population with data from both previous AFLP and RFLP maps resolved 10 linkage groups totalling 544 cM in length. A total of 224 markers were mapped to 168 different loci with an average interval of 3.2 cM.

The last map was the Germany-Edinburgh combined map which was built with data combined from Amanda and Zsuzsanna's populations. It resolved 12 linkage groups with a total length of 424 cM, though linkage groups representing the same chromosome could be identified on the basis of markers shared with previous maps. A total of 377 markers were mapped to 215 different loci with an average interval of 2.0 cM. A summary of five different maps is present in Table 3.3.7.

**Table 3.3.7 Characteristics and comparison of five different *Antirrhinum* maps.**

| Map name | no. of Linkage Groups | no. of loci | no. of markers | total length (cM) | average interval (cM) | population | main marker type* |
|---|---|---|---|---|---|---|---|
| A-Y | 8 | 142 | 194 | 483 | 3.4 | 107 | A+C |
| Z RFLP | 8 | 179 | 264 | 453 | 2.5 | 96 | R+C |
| Z AFLP | 7 | 34 | 34 | 221 | 6.5 | 96 | A |
| Z RFLP+AFLP | 10 | 168 | 224 | 544 | 3.2 | 96 | A+C+R |
| EDI-GER | 12 | 215 | 377 | 424 | 2.0 | 203 | A+C+R |

* marker types, only main markers are listed. A: AFLP, C: CAPS, R: RFLP.

## Discussion

### Chromosome identification

Cytogenetic analysis has been applied to study *A. majus* genome differences. Fluorescent In Situ Hybridisation (FISH) has been used to establish karyotype by anchoring centromeric repeats on the meiotic pachytene chromosome (Zhang *et al.*, 2005). From the FISH analysis, one or two molecular markers were selected from each linkage group to screen *Antirrhinum* transformation-competent artificial chromosome (TAC) library. The TAC clones were labelled as FISH probes and hybridized to pachytene chromosomes of *A. majus* (Figure 3.3.4; Zhang *et al.*, 2005). The maps were compared to the FISH analysis results and show that the linkage groups were similar to the FISH results (Table 3.3.8). The FISH results show that in general, when the molecular markers are mapped close to the end of the chromosome, the physical distance is shorter than the genetic distance. Conversely, markers mapped near the centromere region show physical distances longer than the genetic distance. In Amanda-Yang map (A-Y map), *HIRZ* and *CDC2D* in chromosome 1 are separated by 7.7% of the total genetic distance in the chromosome compared to 5.6% of the physical distance in the FISH result. In this case both genetic and physical distances are very similar to each other. However, two markers *PLE* and *SQUA* were located on chromosome 3. The A-Y map shows these two markers separated by 22% of the total genetic distance of chromosome 3 compared to 67% of the total physical distance in the FISH analysis. This suggests that the region between *PLE* and *SQUA* has less recombination per length of DNA than the region between *HIRZ* and *CDC2*. In the Zuszsanna AFLP-RFLP combined map, four chromosomes could be compared with the FISH result. The results show that only in chromosome 1 is the genetic distance longer than the physical distance from the FISH result. The physical distances in chromosome 3, 6, and 8 were larger than their genetic distances. In the Edinburgh-Germany combined map differences between the genetic distances and the physical distances were also seen. The discrepancy between genetic and physical distance might be caused by two reasons. One is due to reduced recombination rate in the pericentromeric regions of the chromosome. The other is affected by the relatively low density of

alleles that reduced viability when homozygous (Schwarz-Sommer *et al.*, 2003). In no case was TRD found to be due to incompatibility between the *A. molle* allele at one locus and the *A. majus* allele at another.

TRD can affect map construction in several ways. It can cause distorted loci to show spurious linkage or underestimate the distances between closely linked loci (Liu, 1998). It therefore tends to affect calculation of map distances and can cause incorrect fusion or splitting of linkage groups. The linkage groups were constructed by using both codominant and dominant markers together. This has the inherent disadvantage of allowing contradictions and unsafe alignments due to the different degrees of accuracy in estimating recombination frequencies (Ritter *et al.* 1990). The maps using different loci differ in their total length and the number of linkage groups resolved. This could have four reasons: TRD affecting map construction, genotype mis-scoring, different information content of different markers (dominant markers have less information per locus than codominant markers), or conservative scoring of AFLP markers as codominant markers (i.e. as a c h b) which reduces information content and introduces the possibility of mis-scoring, for example h as b, or b as h.

Although mapping was probably affected by these factors, construction of robust linkage maps was possible and the linkage groups consistent with FISH results. This resolved maps that could be used for QTL analysis in the different populations.

## • QUANTITATIVE TRAIT LOCI MAPPING

The genetic maps were constructed from both the Edinburgh and Germany populations, and individuals from both populations were genotyped separately. In order to be able to carry out regression of the trait values onto genotype, QTL Express was used as a method of estimating probabilities of genotypes at positions between markers for regression. The step-wise regression was used, in which the effect of the most significant QTL for a trait was removed, by fixing its most likely position in QTL Express, before identifying the next most likely QTL until no more significant QTL could be detected. Significance as assessed at the 0.95 level using the method of Churchill and Doerge (1994) implemented in QTL Express. Once

all significant QTL had been fixed, the effect of each was determined under conditions where the effects of all others remained fixed.

## 3.4 QTL Mapping for the Edinburgh F2 Population

A total of 63 traits from the Edinburgh F2 population were analysed for significant QTL using QTL Express and the genotype data and map for this population. The results showed 89 QTLs with LOD scores higher than 2.5 for the different traits. The results are summarised below. The most likely position of each QTL is given in cM and the likelihood of a QTL at that position given as both a LOD score and Fisher's F-ratio. The mean trait value for the population was calculated from the regression of the trait value onto genotypes in QTL Express and can differ slightly from the arithmetic mean for the population. The additive effect is defined as half the mean effect of substituting *A. majus* for *A. molle* alleles at the most likely position for the QTL. Negative values indicate that the *A. majus* allele decreases the value of the trait. The dominance effect expresses the mean trait value of heterozygotes relative to the mean of homozygotes. The dominant value can exceed the additive value in magnitude if the alleles show over-dominance (i.e. the heterozygotes have a more extreme value than either homozygote). The percentage variance explained (PVE) by each QTL is estimated from the residual variance after fitting the QTL in QTL Express (see Materials and Methods) and the total PVE is the sum of PVEs for all QTL detected as affecting the trait significantly. Traits with no significant QTL are not listed.

### Edinburgh F2 with Amanda's AFLP Map

### 1. Whole leaf traits

Two QTLs were found for leaf breadth (Lg 3 and 8; Table 3.4), together explaining 30% of the total variance. Leaf area did not show any significant QTL. A single QTL for leaf perimeter was detected towards the end of linkage group 8, accounting for 17% of the total variance (Table 3.4). Detection of QTLs for leaf perimeter and breadth, but not leaf area was surprising as was detection of different QTL for leaf breadth and leaf perimeter because all three traits are strongly correlated.

markers on the genetic maps which might therefore not represent the whole genome. The FISH mapping data also confirmed that the current Antirrhinum map might not cover the full genome (Zhang *et al.*, 2005).

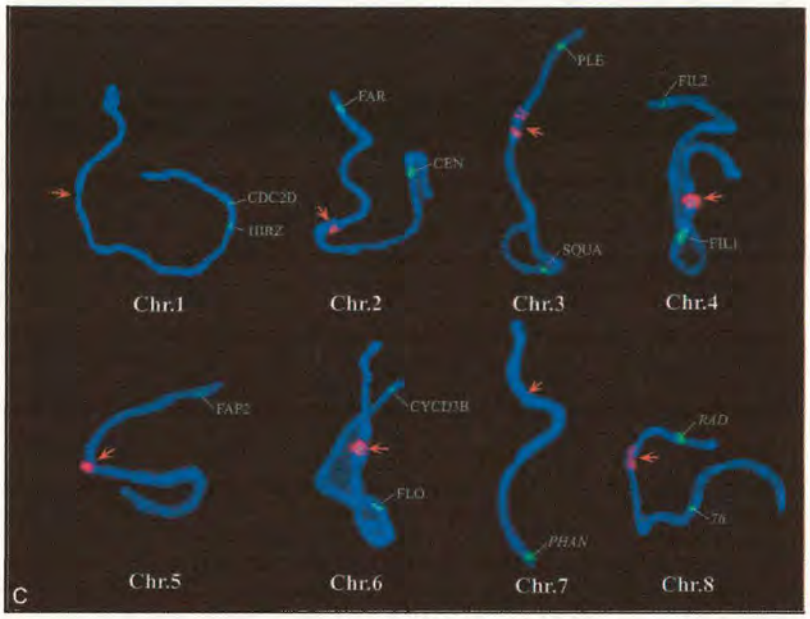



**Figure 3.3.4 Mapping of heterochromatin regions and integration of the chromosome map with the linkage group in Antirrhinum.** The pachytene chromosomes of *A. majus* were probed with different TAC clones (green signals) anchored by different markers as well as CentA1 (red signals). Arrows show the centromere positions (picture reproduced from Zhang et *al.*, 2005).

## Transmission ratio distortion

The potential causes of transmission ratio distortion (TRD) were investigated in the previous *Antirrhinum* map. Interspecific hybrids generally have TRD (Zamir and Tadmor 1986; Fishman *et al.* 2001), and it has been mentioned in *Antirrhinum* studies before (Hoffmann, 1949). TRD results in a failure to get 3:1 or 1:2:1 segregation for markers. Significant TRD was observed for a large part of the genome in the *A. majus* x *A. molle* F2. The reasons for this distortion probably differ for different loci (Schwarz-Sommer *et al.*, 2003). For example, F2 plants homozygous for the region of the chromosome around the self incompatibility locus on LG6 are not recovered because pollen carrying the *S* allele from *A. molle* cannot fertilize a plant also carrying this allele. The effects of this distortion decrease with distance from the *S* locus due to recombination. At other loci, evidence was obtained for the effects of

**Table 3.4 Results of leaf QTL analysis.**

| LINKAGE GROUP | TRAIT | QTL LOCATION | F | LOD | Mean | Additive Effect | Dominance Effect | PVE |
|---|---|---|---|---|---|---|---|---|
| 3 | L-Breadth | 12cM | 8.06 | 3.06 | 28.59 | -1.42 | 3.17 | 16.52 |
| 8 | L-Breadth | 51cM | 6.80 | 2.63 | 27.84 | 2.74 | 0.59 | 13.82 |
| 8 | L-Perimeter | 106cM | 6.98 | 2.71 | 180.27 | 20.43 | -21.56 | 17.1 |

## 2. Adaxial Leaf Epidermis

Three QTLs were found for adaxial leaf cell area (Lg 6 and 8), together explaining 87% of the total variance. Two QTLs were found for leaf cell compactness (Lg 4 and 8), together explaining 40% of the total variance. For cell elongation, three QTLs were found in Lg 1, 6 and 8, together explaining 57% of the total variance. Maximum cell length has two QTLs found in Lg 6 and 8, together explaining 44% of the total variance. A single QTL for cell perimeter was detected towards the top of linkage group 8, accounting for 19% of the total variance (Table 3.4.1). The QTL on Lg 6 at 5 cM and the QTL on Lg 8 around 25-30 cM appear to affect cell area and perimeter (with the *A. molle* allele increasing the value) which are highly correlated traits. It also decreases the mean maximum cell length and elongation and increases cell compactness. This suggests that the QTL both affect polarised cell growth so that an increase in cell size involves a disproportionate elongation of cells. Although leaf cell shape and size appeared to be under strong genetic control, none of the QTL affecting cell shape or size are likely to correspond to the QTL detected as affecting leaf shape or size, suggesting that the major differences in leaf shape and size result from differences in cell number and not cell expansion and that differences in cell shape and size do not cause differences in leaf shape.

**Table 3.4.1 Results of adaxial leaf epidermis QTL analysis.** Black: total explained percentage.

| LINKAGE GROUP | TRAIT | QTL LOCATION | F | LOD | Mean | Additive Effect | Dominance Effect | PVE |
|---|---|---|---|---|---|---|---|---|
| 6 | Cell Area | 5cM | 12.80 | 3.87 | 696.57 | -253.22 | -348.80 | 31.28 |
| 8 | Cell Area | 70cM | 12.05 | 3.70 | 696.57 | -59.09 | -1.21 | 29.31 |
| 8 | Cell Area | 30cM | 12.50 | 4.46 | 415.97 | -53.02 | -3.06 | 26.7 |
| | | | | | | | | ***87.29*** |

**Table 3.4.1 (Cont.)**

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 4 | Compactness | 1cM | 9.25 | 3.45 | 0.62 | -0.01 | 0.03 | 20.00 |
| 8 | Compactness | 25cM | 10.03 | 3.70 | 0.62 | 0.02 | 0.01 | 20.00 |
| | | | | | | | | ***40.00*** |
| 1 | Elongation | 38cM | 15.05 | 5.16 | 2.14 | 0.06 | -0.15 | 25.52 |
| 6 | Elongation | 5cM | 10.47 | 3.82 | 2.14 | -0.15 | -0.20 | 17.24 |
| 8 | Elongation | 5cM | 9.08 | 3.38 | 2.14 | -0.07 | -0.07 | 14.48 |
| | | | | | | | | ***57.24*** |
| 6 | Max_Length | 5cM | 7.22 | 2.78 | 37.36 | -0.29 | -3.74 | 13.16 |
| 8 | Max_Length | 30cM | 15.62 | 5.35 | 37.36 | -3.40 | -0.71 | 30.92 |
| | | | | | | | | ***44.08*** |
| 8 | Perimeter | 25cM | 7.76 | 2.97 | 123.11 | -14.34 | -4.83 | 18.89 |

## 3. Petal Scan

A single QTL for upper petal perimeter was detected towards the top of Lg 8, accounting for 17% of the total variance. Petal maximum breadth show two QTLs found in Lg 8 and 9, together explaining 44% of the total variance. The QTL detected in each case were different. Petal area and maximum length did not show any significant QTL (Table 3.4.2). These results were surprising because petal area, perimeter, length and breadth were highly correlated traits. It is unlikely that any of the QTL detected for petal shape and size correspond to the QTL found to affect leaf shape and size, consistent with the low correlation between these traits.

**Table 3.4.2 Results of petal QTL analysis.** Black: total explained percentage.

| LINKAGE GROUP | TRAIT | QTL LOCATION | F | LOD | Mean | Additive Effect | Dominance Effect | PVE |
|---|---|---|---|---|---|---|---|---|
| 8 | Perimeter | 4cM | 7.01 | 2.72 | 59.37 | 4.85 | 4.62 | 17.17 |
| 8 | Max. Breadth | 25cM | 13.34 | 4.71 | 12.57 | 1.36 | 1.29 | 28.35 |
| 9 | Max. Breadth | 2cM | 7.73 | 2.95 | 12.61 | 1.15 | 0.45 | 15.54 |
| | | | | | | | | ***43.89*** |

## 4. Adaxial petal epidermal cells

Only one single QTL for petal cell shape or size was detected, affecting adaxial petal epidermis cell roundness and located towards the lower of linkage group 1, accounting for 12% of the total variance (Table 3.4.3). Although a QTL affecting leaf cell shape had been detected in this region (38 cM on Lg 1), the two QTL had opposite effects on cell shape in the two organs with the *A. molle* chromosome acting dominantly to promote leaf cell elongation and petal cell roundness, suggesting that they were not the same. The QTL affecting petal cell shape did not correspond to any of the QTL affecting petal size or shape, suggesting that these traits were under independent genetic control, as previously suggested by the low correlation between petal traits and petal cell traits.

**Table 3.4.3 Results of adaxial petal epidermis QTL analysis.**

| LINKAGE GROUP | TRAIT | QTL LOCATION | F | LOD | Mean | Additive Effect | Dominance Effect | PVE |
|---|---|---|---|---|---|---|---|---|
| 1 | Roundness | 49cM | 6.52 | 2.63 | -20.48 | 0.52 | -32.97 | 11.74 |

## 5. Flower

Two QTLs were found corresponding to pedicel length (Lg3 and 6), together explaining 35% of the total variance. Two QTLs were found for petal tube length (Lg7 and 8), both QTLs explaining 40% of the total variance. There were two QTLs found for mid-lobe width (Lg7 and 8), together explaining 30% of the total variance. Three QTLs were found for the gibba length (Lg 1, 4 and 8), together explaining 65% of the total variance. In upper petal length, three QTLs were found in Lg 5, 7 and 8, together explaining 65% of the variance. Two QTLs for the lower lip width were found in Lg 4 and 7, explaining up to 45% of the total variance. Four QTLs were found for middle lip height (Lg 3, 4, 7 and 8), together explaining up to 75% of the total variance. A single QTL was found for style length at the end of Lg 4, accounting for 22% of the total variance. Two QTLs were found for short anther filament length (Lg 4 and 8), together explaining 37% of the total variance. No significant QTL was detected for tube height or long anther filament length (Table 3.4.4). In several cases the QTL were likely to affect more than one trait, for example the first part of Lg 7 affected several petal traits in the same way (*A. molle* allele

increasing their sizes) and in most cases the traits sharing QTLs were significantly correlated (see Figure 3.1.4). For instance, Lg 7 contains a QTL at which the *A. molle* allele increases mid lobe width (F-4), upper petal side length (F-6) and petal lip width (F-7), traits that are significantly correlated (see Table 3.1.4).

**Table 3.4.4 Results of flower QTL analysis.** Black: total explained percentage.

| LINKAGE GROUP | TRAIT* | QTL LOCATION | F | LOD | Mean | Additive Effect | Dominance Effect | PVE |
|---|---|---|---|---|---|---|---|---|
| 3 | F-1 | 18cM | 7.06 | 2.72 | 9.40 | -1.74 | -0.62 | 14.76 |
| 6 | F-1 | 37cM | 9.73 | 3.61 | 9.40 | -1.98 | -0.30 | 20.96 |
| | | | | | | | | ***35.72*** |
| 7 | F-2 | 6cM | 8.38 | 3.15 | 12.1 | -0.53 | -0.01 | 15.69 |
| 8 | F-2 | 9cM | 12.97 | 4.57 | 12.11 | 0.41 | 0.70 | 25.25 |
| | | | | | | | | ***40.94*** |
| 7 | F-4 | 14cM | 10.33 | 3.76 | 11.24 | -0.74 | 0.42 | 15.93 |
| 8 | F-4 | 3cM | 8.45 | 3.16 | 11.12 | 0.72 | 1.16 | 13.13 |
| | | | | | | | | ***29.06*** |
| 1 | F-5 | 21cM | 10.31 | 3.77 | 8.83 | 0.86 | -0.61 | 17.57 |
| 4 | F-5 | 32cM | 17.12 | 5.71 | 8.54 | 0.66 | 0.15 | 31.81 |
| 8 | F-5 | 61cM | 8.48 | 3.19 | 8.54 | 0.09 | -0.65 | 14.81 |
| | | | | | | | | ***64.19*** |
| 5 | F-6 | 25cM | 9.25 | 3.44 | 13.53 | 0.77 | 0.34 | 11.71 |
| 7 | F-6 | 17cM | 17.07 | 5.7 | 13.61 | -0.65 | 1.09 | 21.46 |
| 8 | F-6 | 30cM | 23.95 | 7.37 | 13.53 | 1.05 | 1.45 | 32.58 |
| | | | | | | | | ***65.75*** |
| 4 | F-7 | 35cM | 13.9 | 4.84 | 5.26 | 0.59 | -0.42 | 25.18 |
| 7 | F-7 | 36cM | 10.67 | 3.88 | 5.28 | -0.4 | 0.68 | 18.84 |
| | | | | | | | | ***44.02*** |
| 3 | F-8 | 15cM | 9.95 | 3.64 | 10.03 | -1.4 | 0.96 | 12.21 |
| 4 | F-8 | 16cM | 17.44 | 5.75 | 10.03 | -1.51 | -0.48 | 22.43 |
| 7 | F-8 | 9cM | 13.69 | 4.74 | 10.03 | -1.01 | 1.27 | 17.32 |
| 8 | F-8 | 0cM | 18.87 | 6.11 | 9.99 | 1.69 | 1.64 | 23.99 |
| | | | | | | | | ***75.95*** |
| 4 | F-9 | 2cM | 9.75 | 3.62 | 13.92 | -0.7 | 0.62 | 22.08 |
| | | | | | | | | ***22.08*** |
| 4 | F-11 | 9cM | 6.77 | 2.62 | 17.56 | -0.57 | -0.37 | 13.43 |
| 8 | F-11 | 28cM | 11.25 | 4.08 | 17.56 | 0.71 | 0.61 | 23.84 |
| | | | | | | | | ***37.27*** |

* **F-1**: pedicel length. **F-2**: flower tube length. **F-4**: mid-lobe width. **F-5**: gibba length. **F-6**: upper petal side length. **F-7**: lower lip width. **F-8**: height from upper petal to lower lip. **F-9**: style length. **F-11**: short anther filament length. (see flower measurement in MATERIALS AND METHODS)

## 6. Pigment

Pigmentation in different parts of the flower was analysed. Three QTLs were found for anthocyanin content in upper petals, measured after extraction (Lg 3 and 7), together explaining up to 65% of the total variance. Two QTLs were found for anthocyanin distribution in the flower central lips (Lg 3 and 7), both QTLs together explaining 60% of the total variance. These are likely to be the same QTL as those affecting anthocyanin content. Only one single QTL was detected for yellow pigment expression in the flower central lips (Lg 4), explaining 30% of the total variance (Table 3.4.5). This locus has no detectable effect on anthocyanin content, suggesting that levels and patterns of the two pigments are under independent genetic control. The locus on Lg 3 is likely to correspond to *ROSEA* (*ROS*) which maps to this position. *ROS* encodes MYB transcripton factors that promote expression of the anthocyanin biosynthetic pathway (Cathie Martin, personal communication) and has been shown to be responsible for differences in pigment intensity between other *Antirrhinum* species (Schwinn, 1971; Stubbe, 1964). One of the loci in Lg 7 might also correspond to the *VENOSA* (*VE*) locus which maps to this Lg and encodes another MYB transcription factor that also promotes expression of anthocyanin biosynthetic genes (Cathie Martin, personal communication) and is know to vary between species (Stubbe, 1964). *VE* allele in *A. majus* allows strong pigmentation of the flower, *VE* is more likely to correspond to the QTL close to the top of Lg 7 at which the *A. majus* allele increases anthocyanin content. At least one locus, *SULFUREA* (*SULF*), is known to affect distribution of yellow aurone pigments in *Antirrhinum* flowers and might correspond to the locus detected in Lg4. However *SULF* has not been mapped and so this could not be tested.

**Table 3.4.5 Results of pigment QTL analysis.** Black: total explained percentage.

| LINKAGE GROUP | TRAIT* | QTL LOCATION | F | LOD | Mean | Additive Effect | Dominance Effect | PVE |
|---|---|---|---|---|---|---|---|---|
| 3 | anthocyanin | 8cM | 19.09 | 6.16 | 0.26 | 0.4 | 0.23 | 26.42 |
| 7 | anthocyanin | 39cM | 11.91 | 4.23 | 0.21 | -0.84 | -0.81 | 15.5 |
| 7 | anthocyanin | 11cM | 14.77 | 5.04 | 0.26 | 0.4 | 0.01 | 20.13 |
| | | | | | | | | ***62.05*** |
| 3 | Red | 11cM | 29.15 | 8.59 | 1.66 | 1.55 | 1.26 | 49.05 |
| 7 | Red | 11cM | 6.50 | 2.53 | 1.74 | 0.67 | 0.04 | 9.62 |
| | | | | | | | | ***58.67*** |
| 4 | Yellow | 4cM | 13.82 | 4.88 | 0.9 | 0.43 | 0.27 | 30.64 |

* **anthocyanin**: extracted from upper petal with extraction buffer. **Red**: anthocyanin expression in the middle lobe judged by eye. **Yellow**: the yellow pigment expression on the middle lobe. (see flower measurement in MATERIALS AND METHODS). Red PVE: total explained percentage variance.

## 7. Other Growth Traits

Three QTLs were detected for branch number (Lg 2, 4 and 7), together explaining about 70% of the total variance. A single QTL was found for flowering time (Lg 1), explaining 36% of the variance. Two QTLs for first flowering node were found (Lg 4 and 7), explaining 55% of the variance. First flowering node is a measure of flowering time that takes differences in the rate of plant development into account. Three QTLs were found for plant total length (Lg 1, 2 and 7), together explaining about 70% of the total variance. Two QTLs explaining 40% of the node diameter variance were found in Lg 6 and 7 (Table 3.4.6). Because the same QTL were not detected for flowering time and flowering node, the flowering time QTL (Lg 1) might affect the overall rate of plant development. Consistent with this, the same QTL affects total plant length in the same way with the *A. majus* allele decreasing days to flower and plant length without apparently affecting the number of nodes formed before flowering. Several other traits were likely to be affected by the same QTL. The QTL on Lg 2 affected total plant length and branch number in the same way (the *A. majus* allele increasing both traits), consistent with the strong correlation between these traits. It suggests that taller plants have more opportunity to branch or that the same gene promotes growth of the main shoot axis and lateral branches.

Similarly the QTL on Lg 7 affected total plant length, branch number, first flowering node and node diameter with the *A. majus* allele increasing the trait values. This suggests that the QTL controls the overall rate of vegetative growth and is consistent with correlations between these traits.

**Table 3.4.6 Results of other growth traits QTL analysis.** Black: total explained percentage.

| LINKAGE GROUP | TRAIT | QTL LOCATION | F | LOD | Mean | Additive Effect | Dominance Effect | PVE |
|---|---|---|---|---|---|---|---|---|
| 2 | branch-no | 7cM | 11.98 | 4.25 | 17.84 | 2.14 | 3.54 | 12.66 |
| 4 | branch-no | 33cM | 7.78 | 2.94 | 17.47 | 2.06 | 0.39 | 7.7 |
| 7 | branch-no | 14cM | 42.44 | 10.77 | 18.07 | 4.13 | -1.9 | 47.48 |
| | | | | | | | | ***67.84*** |
| 1 | days-to-flower | 28cM | 17.05 | 5.74 | 80.37 | -4.27 | -2.95 | 36.4 |
| 4 | flowering-node | 2cM | 6.75 | 2.61 | 11.99 | 1.78 | -0.25 | 9.54 |
| 7 | flowering-node | 14cM | 19.52 | 6.43 | 11.99 | 2.99 | -1.58 | 44.2 |
| | | | | | | | | ***53.74*** |
| 6 | node-diameter | 43cM | 12.3 | 4.4 | 2.46 | 0.18 | 0.27 | 20.38 |
| 7 | node-diameter | 18cM | 10.92 | 3.98 | 2.44 | 0.22 | 0.03 | 17.91 |
| | | | | | | | | ***38.29*** |
| 1 | total-length | 28cM | 20.82 | 6.64 | 31.03 | -5.52 | 0.66 | 22.22 |
| 2 | total-length | 12cM | 17.18 | 5.73 | 31.03 | 3.78 | 3.68 | 18.14 |
| 7 | total-length | 15cM | 25.17 | 7.64 | 31.03 | 4.3 | -1.93 | 27.1 |
| | | | | | | | | ***67.46*** |

## 8. Hairs

The density of epidermal hairs (trichomes) of different morphology (their length and the presence or absence of a multicellular gland at the tip) was counted for the F2 generation. A single QTL was found to affect the density of glandular hairs in the abaxial leaf surface (Lg8), explaining up to 50% of the total variance. Three QTLs were found for the density of very long aglandular hairs on the abaxial leaf surface (Lg 6 and 8), together explaining 99% of the total variance. For the density of all abaxial aglandular hairs, the same two QTLs were found in Lg 6 and 8, both QTLs together explaining 98% of the total variance. Therefore the QTL around 31 cM on Lg 8 appeared to affect the density of both glandular and aglandular trichomes, with the *A. majus* allele having an additive effect to promote glandular trichome density and a dominant effect to promote aglandular density. However the strongest effect was that of the QTL on Lg 6 at which the *A. majus* allele acted as a dominant suppressor of aglandular trichome development.

**Table 3.4.7 Results of hair QTL analysis.** Black: total explained percentage.

| LINKAGE GROUP | TRAIT | QTL LOCATION | F | LOD | Mean | Additive Effect | Dominance Effect | PVE |
|---|---|---|---|---|---|---|---|---|
| 8 | DL_totG | 31cM | 16.43 | 4.72 | 21.04 | 20.45 | -10.07 | 50.7 |
| 6 | DL_#VLA | 5cM | 73.41 | 10.23 | 145.99 | -149.11 | -147.7 | 71.61 |
| 8 | DL_#VLA | 72cM | 10.81 | 3.35 | 145.99 | 7.25 | 6.96 | 9.7 |
| 8 | DL_#VLA | 32cM | 18.59 | 4.88 | 145.99 | -8.6 | 11.46 | 17.39 |
| | | | | | | | | ***98.7*** |
| 6 | DL_totA | 5cM | 74.08 | 10.27 | 146.26 | -150.11 | -147.33 | 74.01 |
| 8 | DL_totA | 31cM | 25.89 | 5.99 | 145.45 | -6.04 | 12.3 | 23.95 |
| | | | | | | | | ***97.96*** |

***DL**: abaxial leaf, **G**: glandular trichome, **A**: aglandular trichome, and **VL**: very long trichome.

## Discussion

Comparing all QTL results from different vegetative and reproductive traits, flowering time and plant length have QTL mapping to the same region in Lg1. Correlation analysis had predicted that these two traits were controlled by the same genes. Branching and plant length were also highly correlated and QTL results showed both traits were controlled by the same gene in Lg2. Style length and anther filament length share QTLs as expected from their correlations. In Lg 6, where gene *CYC* and *MIXTA-like2* (*ML2*) are located a QTL was detected that affected epidermal cell area, maximum length, and cell elongation in the adaxial leaf epidermis. The same region affected trichome densities in the abaxial leaf. *MIXTA* and *MIXTA-like* genes have been found related to epidermis cell differences. The ectopic expression of the *MIXTA* gene from *A. majus* in tobacco results in the formation of excess numbers of multicellular trichomes in the leaf epidermis and a concomitant reduction in stomatal density (Glover, 2000). Traits from flower and petal size have QTLs in the same region in Lg 7 and 8. Flower tube length, and lobe width have QTL in the same region as petal length and width; however, these reproductive traits did not show high correlation. Regions where QTLs affected more than one trait are summarised in Figure 3.4.

**Table 3.4.8 Comparison of QTLs for different vegetative and reproductive traits.**

| LINKAGE GROUP | TRAIT | QTL LOCATION | F | LOD | Mean | Additive Effect | Dominance Effect | PVE |
|---|---|---|---|---|---|---|---|---|
| 1 | F-5 | 21cM | 10.31 | 3.77 | 8.83 | 0.86 | -0.61 | 17.57 |
| 1 | day-to-flower | 28cM | 17.05 | 5.74 | 80.37 | -4.27 | -2.95 | 36.4 |
| 1 | total-length | 28cM | 20.82 | 6.64 | 31.03 | -5.52 | 0.66 | 22.22 |
| 1 | Adax-leaf-Elongation | 38cM | 15.05 | 5.16 | 2.14 | 0.06 | -0.15 | 25.52 |
| 1 | Adax-petal-Roundness | 49cM | 6.52 | 2.63 | -20.48 | 0.52 | -32.97 | 11.74 |
| 2 | branch-no | 7cM | 11.98 | 4.25 | 17.84 | 2.14 | 3.54 | 12.66 |
| 2 | total-length | 12cM | 17.18 | 5.73 | 31.03 | 3.78 | 3.68 | 18.14 |
| 3 | anthocyanin | 8cM | 19.09 | 6.16 | 0.26 | 0.4 | 0.23 | 26.42 |
| 3 | Red | 11cM | 29.15 | 8.59 | 1.66 | 1.55 | 1.26 | 49.05 |
| 3 | L-Breadth | 12cM | 8.06 | 3.06 | 28.59 | -1.42 | 3.17 | 16.52 |
| 3 | F-8 | 15cM | 9.95 | 3.64 | 10.03 | -1.4 | 0.96 | 12.21 |
| 3 | F-1 | 18cM | 7.06 | 2.72 | 9.4 | -1.74 | -0.62 | 14.76 |
| 4 | Adax-leaf-Compactness | 1cM | 9.25 | 3.45 | 0.62 | -0.01 | 0.03 | 20 |
| 4 | F-9 | 2cM | 9.75 | 3.62 | 13.92 | -0.7 | 0.62 | 22.08 |
| 4 | flowering-node | 2cM | 6.75 | 2.61 | 11.99 | 1.78 | -0.25 | 9.54 |
| 4 | Yellow | 4cM | 13.82 | 4.88 | 0.9 | 0.43 | 0.27 | 30.64 |
| 4 | F-11 | 9cM | 6.77 | 2.62 | 17.56 | -0.57 | -0.37 | 13.43 |
| 4 | F-8 | 16cM | 17.44 | 5.75 | 10.03 | -1.51 | -0.48 | 22.43 |
| 4 | F-5 | 32cM | 17.12 | 5.71 | 8.54 | 0.66 | 0.15 | 31.81 |
| 4 | branch-no | 33cM | 7.78 | 2.94 | 17.47 | 2.06 | 0.39 | 7.7 |
| 4 | F-7 | 35cM | 13.9 | 4.84 | 5.26 | 0.59 | -0.42 | 25.18 |
| 5 | F-6 | 25cM | 9.25 | 3.44 | 13.53 | 0.77 | 0.34 | 11.71 |
| 6 | Adax-leaf-Cell Area | 5cM | 12.8 | 3.87 | 696.57 | -253.22 | -348.8 | 31.28 |
| 6 | Adax-leaf-Elongation | 5cM | 10.47 | 3.82 | 2.14 | -0.15 | -0.2 | 17.24 |
| 6 | Adax-leaf-Max_Length | 5cM | 7.22 | 2.78 | 37.36 | -0.29 | -3.74 | 13.16 |
| 6 | DL_#VLA | 5cM | 73.41 | 10.23 | 145.99 | -149.11 | -147.7 | 71.61 |
| 6 | DL_totA | 5cM | 74.08 | 10.27 | 146.26 | -150.11 | -147.33 | 74.01 |
| 6 | F-1 | 37cM | 9.73 | 3.61 | 9.4 | -1.98 | -0.3 | 20.96 |
| 6 | node-diameter | 43cM | 12.3 | 4.4 | 2.46 | 0.18 | 0.27 | 20.38 |

**Table 3.4.8 (Cont.)**

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 7 | F-2 | 6cM | 8.38 | 3.15 | 12.1 | -0.53 | -0.01 | 15.69 |
| 7 | F-8 | 9cM | 13.69 | 4.74 | 10.03 | -1.01 | 1.27 | 17.32 |
| 7 | anthocyanin | 11cM | 14.77 | 5.04 | 0.26 | 0.4 | 0.01 | 20.13 |
| 7 | Red | 11cM | 6.5 | 2.53 | 1.74 | 0.67 | 0.04 | 9.62 |
| 7 | branch-no | 14cM | 42.44 | 10.77 | 18.07 | 4.13 | -1.9 | 47.48 |
| 7 | F-4 | 14cM | 10.33 | 3.76 | 11.24 | -0.74 | 0.42 | 15.93 |
| 7 | flowering-node | 14cM | 19.52 | 6.43 | 11.99 | 2.99 | -1.58 | 44.2 |
| 7 | total-length | 15cM | 25.17 | 7.64 | 31.03 | 4.3 | -1.93 | 27.1 |
| 7 | F-6 | 17cM | 17.07 | 5.7 | 13.61 | -0.65 | 1.09 | 21.46 |
| 7 | node-diameter | 18cM | 10.92 | 3.98 | 2.44 | 0.22 | 0.03 | 17.91 |
| 7 | F-7 | 36cM | 10.67 | 3.88 | 5.28 | -0.4 | 0.68 | 18.84 |
| 7 | anthocyanin | 39cM | 11.91 | 4.23 | 0.21 | -0.84 | -0.81 | 15.5 |
| 8 | F-8 | 0cM | 18.87 | 6.11 | 9.99 | 1.69 | 1.64 | 23.99 |
| 8 | F-4 | 3cM | 8.45 | 3.16 | 11.12 | 0.72 | 1.16 | 13.13 |
| 8 | Petal-Perimeter | 4cM | 7.01 | 2.72 | 59.37 | 4.85 | 4.62 | 17.17 |
| 8 | Adax-leaf-Elongation | 5cM | 9.08 | 3.38 | 2.14 | -0.07 | -0.07 | 14.48 |
| 8 | F-2 | 9cM | 12.97 | 4.57 | 12.11 | 0.41 | 0.7 | 25.25 |
| 8 | Adax-leaf-Compactness | 25cM | 10.03 | 3.7 | 0.62 | 0.02 | 0.01 | 20 |
| 8 | Adax-leaf-Perimeter | 25cM | 7.76 | 2.97 | 123.11 | -14.34 | -4.83 | 18.89 |
| 8 | Petal-Max. Breadth | 25cM | 13.34 | 4.71 | 12.57 | 1.36 | 1.29 | 28.35 |
| 8 | F-11 | 28cM | 11.25 | 4.08 | 17.56 | 0.71 | 0.61 | 23.84 |
| 8 | Adax-leaf-Cell Area | 30cM | 12.5 | 4.46 | 415.97 | -53.02 | -3.06 | 26.7 |
| 8 | Ad-leaf-Max_Length | 30cM | 15.62 | 5.35 | 37.36 | -3.4 | -0.71 | 30.92 |
| 8 | F-6 | 30cM | 23.95 | 7.37 | 13.53 | 1.05 | 1.45 | 32.58 |
| 8 | DL_totA | 31cM | 25.89 | 5.99 | 145.45 | -6.04 | 12.3 | 23.95 |
| 8 | DL_totG | 31cM | 16.43 | 4.72 | 21.04 | 20.45 | -10.07 | 50.7 |
| 8 | DL_#VLA | 32cM | 18.59 | 4.88 | 145.99 | -8.6 | 11.46 | 17.39 |
| 8 | L-Breadth | 51cM | 6.8 | 2.63 | 27.84 | 2.74 | 0.59 | 13.82 |
| 8 | F-5 | 61cM | 8.48 | 3.19 | 8.54 | 0.09 | -0.65 | 14.81 |
| 8 | Ad-leaf-Cell Area | 70cM | 12.05 | 3.7 | 696.57 | -59.09 | -1.21 | 29.31 |
| 8 | DL_#VLA | 72cM | 10.81 | 3.35 | 145.99 | 7.25 | 6.96 | 9.7 |
| 8 | L-Perimeter | 106cM | 6.98 | 2.71 | 180.27 | 20.43 | -21.56 | 17.1 |
| 9 | P-Max. Breadth | 2cM | 7.73 | 2.95 | 12.61 | 1.15 | 0.45 | 15.54 |

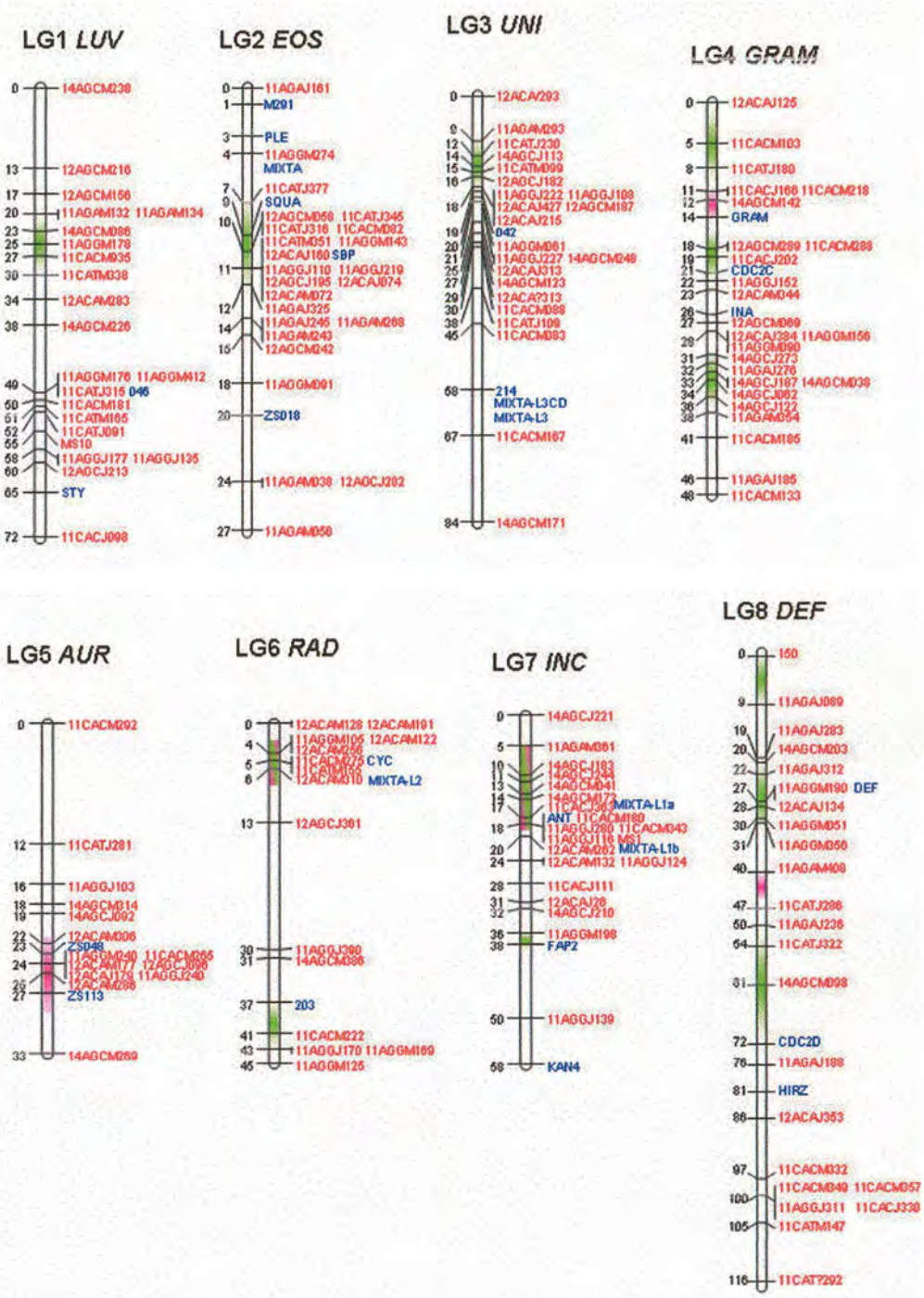



**Figure 3.4 QTL distribution along the linkage groups.** The distribution of QTL affecting both vegetative and reproductive traits is shown in green. QTL region for PCs are shown in pink.

## 9. PCA QTL Results

For analysis of leaf shape and size variance, a shape model with 14 points along the leaf outline was applied (see Materials and Methods). Principal Component Analysis (PCA) was used to estimate the variance of each of the 14 points as 3-4 principal components (PCs) that described more than 95% of the whole shape and size variance. The value of each PC was calculated for each individual. The PC value from each individual was used for QTL analysis.

Principal Components (PCs) were analysed in two different models using Langlade's method: one was the trait data for the *A. majus* x *A. molle* F2 population in the shape model made from these data (*molle* in *molle* model - *m/m*), the other was the *A. majus* x *A. molle* data in a model constructed from an F2 population of *A. majus* x *A. charidemi* (*molle* data in *charidemi* model - *m/c*). In the *m/m* analysis, eleven QTLs were found for PC1 to PC4. However, no QTL was found for PC3, which captured variation in the angle of the petiole relative to the leaf blade. One possible reason might be that variation in the leaf petiole angle is the result of mis-aligning blade and petiole when flattening leaves and is therefore not expected to have a genetic component.

In *m/m* PC analysis, five QTLs were detected for PC1 (Lg 2, 7 and 8) which shows the main variance in leaf size and shape; five QTLs together explaining 60% of the total variance. Four QTLs were found for PC2 (Lg 4, 5, 8 and 9), together explaining 32% of the total variance. There was no significant QTL found for PC3. Two QTL were found for PC4 (Lg 6 and 7), which together explain up to 25% of the total variance (Table 3.4.9). QTLs for different PCs are different in the m/m PC analysis suggesting that they have a different genetic basis.

**Table 3.4.9 Results of *molle* in *molle* model *(m/m)* PCs QTL analysis.** Black: total explained percentage.

| LINKAGE GROUP | TRAIT | QTL LOCATION | F | LOD | Mean | Additive Effect | Dominance Effect | PVE |
|---|---|---|---|---|---|---|---|---|
| 2 | PC1 | 20cM | 13.52 | 5.14 | -35.19 | 73.78 | -67.9 | 17.44 |
| 2 | PC1 | 9cM | 10.08 | 3.96 | -35.19 | -42.1 | 91.3 | 12.65 |
| 7 | PC1 | 27cM | 9.47 | 3.74 | -34.59 | 55.8 | 45.2 | 11.71 |
| 8 | PC1 | 110cM | 6.93 | 2.8 | -30.53 | 49.03 | -38.1 | 8.24 |
| 8 | PC1 | 23cM | 7.66 | 3.07 | -35.19 | 51.14 | 6.83 | 9.28 |
| | | | | | | | | ***59.32*** |
| 4 | PC2 | 12cM | 11.21 | 4.36 | -6.26 | -12.8 | -1.21 | 10.85 |
| 5 | PC2 | 27cM | 5.51 | 2.26 | -6.26 | -6.5 | 5.19 | 4.79 |
| 8 | PC2 | 42cM | 10.22 | 4.01 | -5.3 | -12.5 | 6.37 | 9.76 |
| 9 | PC2 | 2cM | 7.59 | 3.05 | -6.26 | -9.98 | -3.71 | 7 |
| | | | | | | | | ***32.4*** |
| 6 | PC4 | 6cM | 7.09 | 2.87 | 11.85 | -16.3 | -11.5 | 9.6 |
| 7 | PC4 | 17cM | 11.33 | 4.42 | 15.73 | 8.41 | -2.3 | 15.57 |
| | | | | | | | | ***25.17*** |

The other PC analysis was done with *molle* data in the *charidemi* model (*m/c*). The result in the *m/c* model showed a total of eight QTLs affecting three different PCs. PC1 explains 65% of the total leaf variance, however there was no significant QTL found for PC1. PC2 explains 20% of the total leaf variance, and there were three QTLs found for PC2 (Lg 3, 4 and 8), explaining 40% of the total variance. Four QTLs were found for PC3 (Lg 3, 4, 5 and 7), together explaining up to 40% of the total variance. A single QTL was detected for PC4 in Lg 6, explaining 10% of the total variance. PC5 only explained 2% of the total leaf variance, and there was no significant QTL detected for PC5. Different QTLs were found for different PCs and PCs did not share any significant QTLs (Table 3.4.10).

**Table 3.4.10 Results of *molle* data in the *charidemi* model *(m/c)* PCs QTL analysis.** Black: total explained percentage.

| LINKAGE GROUP | TRAIT | QTL LOCATION | F | LOD | Mean | Additive Effect | Dominance Effect | PVE |
|---|---|---|---|---|---|---|---|---|
| 3 | PC2 | 15cM | 6.72 | 2.73 | -35.28 | -5.25 | -13.46 | 6.92 |
| 4 | PC2 | 12cM | 17.56 | 6.49 | -35.28 | -15.1 | -2.58 | 20.03 |
| 8 | PC2 | 43cM | 11.49 | 4.47 | -34.95 | -14.65 | 4.51 | 12.68 |
| | | | | | | | | ***39.63*** |

**Table 3.4.10 (Cont.)**

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 3 | PC3 | 24cM | 9.44 | 3.73 | -9.85 | 7.35 | -7.39 | 10.57 |
| 4 | PC3 | 21cM | 6.69 | 2.71 | -9.85 | 4.83 | 7.46 | 7.12 |
| 5 | PC3 | 21cM | 9.59 | 3.79 | -9.12 | 7.68 | -1.56 | 10.67 |
| 7 | PC3 | 14cM | 11.82 | 4.57 | -10.06 | -7.76 | 5.71 | 13.48 |
| | | | | | | | | ***41.84*** |
| 6 | PC4 | 6cM | 6.18 | 2.53 | 26.44 | -18.66 | -20.83 | 9.3 |

## Discussion

Within each model there were no similarities between the QTLs for different PCs, however, there were similarities between two different models. Two QTLs for PC2 were found at the same location (Lg 4 and 8) for both models, this suggests that the shape differences captured by PC2 were similar in both models and possibly that leaf shape is controlled by the same genes in *A. molle* and *A. charidemi*. Although two species have the same QTL for PC2 at Lg 4, the result shows that QTL in the *m/c* model has double the effect detected in the *m/m* model (10%). A single QTL for *m/c* PC3 was found in the same region as a QTL for *m/m* PC2 on Lg 5. A single QTL was also found on Lg 6 for PC4 in both models and a single QTL was found for *m/c* PC3 and *m/m* PC4 on Lg 7. PC1 captures mainly variance in leaf size, with a limited shape component; however, the result did not detect any QTL affecting PC1 in both models. PC2 describes mainly variance in leaf shape in both models and allows detection of the same QTL affecting leaf shape in both models. In Lg 5, QTLs have been detected for PC2 in *m/m* model and PC3 for *m/c* model, that were at the same region are controlled by the same genes. For PC4, which describes mainly the variance in petiole length, QTLs were detected at the same region on Lg 6 for both models, suggesting that both models were able to describe the effects of genes influencing petiole length. PC3 from the *m/c* model detected a single QTL at the same region as PC4 for the *molle* model in Lg 7, both QTL explaining ~15% of the total variance. The comparisons of similarities for QTLs between two different models are present below. The regions where QTLs were distributed are presented in Figure 3.4.

**Table 3.4.11 Comparison for QTL analysis for PCs under different models.**

| LINKAGE GROUP | TRAIT | QTL LOCATION | F | LOD | Mean | Additive Effect | Dominance Effect | PVE |
|---|---|---|---|---|---|---|---|---|
| 2 | *m/m*_PC1 | 20cM | 13.52 | 5.14 | -35.19 | 73.78 | -67.9 | 17.44 |
| 2 | *m/m*_PC1 | 9cM | 10.08 | 3.96 | -35.19 | -42.1 | 91.3 | 12.65 |
| 3 | *m/c*_PC2 | 15cM | 6.72 | 2.73 | -35.28 | -5.25 | -13.46 | 6.92 |
| 3 | *m/c*_PC3 | 24cM | 9.44 | 3.73 | -9.85 | 7.35 | -7.39 | 10.57 |
| **4** | ***m/m*_PC2** | **12cM** | **11.21** | **4.36** | **-6.26** | **-12.8** | **-1.21** | **10.85** |
| **4** | ***m/c*_PC2** | **12cM** | **17.56** | **6.49** | **-35.28** | **-15.1** | **-2.58** | **20.03** |
| 4 | *m/c*_PC3 | 21cM | 6.69 | 2.71 | -9.85 | 4.83 | 7.46 | 7.12 |
| **5** | ***m/c*_PC3** | **21cM** | **9.59** | **3.79** | **-9.12** | **7.68** | **-1.56** | **10.67** |
| **5** | ***m/m*_PC2** | **27cM** | **5.51** | **2.26** | **-6.26** | **-6.5** | **5.19** | **4.79** |
| **6** | ***m/m*_PC4** | **6cM** | **7.09** | **2.87** | **11.85** | **-16.3** | **-11.5** | **9.6** |
| **6** | ***m/c*_PC4** | **6cM** | **6.18** | **2.53** | **26.44** | **-18.66** | **-20.83** | **9.3** |
| **7** | ***m/c*_PC3** | **14cM** | **11.82** | **4.57** | **-10.06** | **-7.76** | **5.71** | **13.48** |
| **7** | ***m/m*_PC4** | **17cM** | **11.33** | **4.42** | **15.73** | **8.41** | **-2.3** | **15.57** |
| 7 | *m/m*_PC1 | 27cM | 9.47 | 3.74 | -34.59 | 55.8 | 45.2 | 11.71 |
| 8 | *m/m*_PC1 | 110cM | 6.93 | 2.8 | -30.53 | 49.03 | -38.1 | 8.24 |
| 8 | *m/m*_PC1 | 23cM | 7.66 | 3.07 | -35.19 | 51.14 | 6.83 | 9.28 |
| **8** | ***m/m*_PC2** | **42cM** | **10.22** | **4.01** | **-5.3** | **-12.5** | **6.37** | **9.76** |
| **8** | ***m/c*_PC2** | **43cM** | **11.49** | **4.47** | **-34.95** | **-14.65** | **4.51** | **12.68** |
| 9 | *m/m*_PC2 | 2cM | 7.59 | 3.05 | -6.26 | -9.98 | -3.71 | 7 |

## 3.5 QTL Mapping with Combined Maps

### Germany F2 & F3 Leaf Traits Mapping With RFLP_AFLP Combined Map

A genetic map combining information from the RFLP and AFLP data from the German F2 population was built to analyse QTL with more genetic information. Thus both German F2 and F3 can be analysed with the same map to compare the mapping results. The result shows that there were three significant QTLs found for the F2 and 6 significant QTLs found for the F3 population. Two QTLs were found for F2 leaf area (Lg 3 and 8), together explaining up to 40% of the total variance. A single QTL was detected for F2 leaf breadth (Lg 4), explaining 20% of the total variance. Three QTLs were

found for F2 leaf length (Lg 1, 3 and 8), together explaining 45% of the total variance. In the F3 population, a single QTL had been found at the same location for both leaf area and breadth (Lg 8), explaining 15% of the total variance in leaf area, and 25% of the variation in leaf breadth. Two QTLs were detected for F3 leaf length (Lg 1 and 8), together explaining 30% of the total variance. For F3 leaf perimeter, there were two QTL found (Lg 1 and 8), together explaining up to 30% of the total variance (Table 3.5.2).

Comparing the results from the German F2 and F3 populations revealed similarities in different regions. A leaf length QTL was found in the same region of Lg1 for both populations. Leaf area and length were found to be controlled by a gene in Lg3 in the F2 population, but no significant QTL was detected in this region for the F3. QTLs for leaf area and length from F2 and F3 were also detected at the top region of Lg8. The analysis of the F3 population was expected to be more powerful, because it involved measuring multiple members of each family, which should have reduced the variance due to environment and allowed more sensitive detection of QTL. However, for leaf area and length it detected fewer QTL that explained less of the total variance. One reason for this is that QTL that were heterozygous in the F2 would have segregated in the F3 and therefore the mean of the F3 family might not have provided a good estimate of the trait value of the F2 parent, which was used in QTL analysis.

**Table 3.5.2 Results of both German F2 and F3 QTL analysis with Zsuzsanna's AFLP_RFLP map.** Black: total explained percentage.

| LINKAGE GROUP | TRAIT | QTL LOCATION | F | LOD | Mean | Additive Effect | Dominance Effect | PVE |
|---|---|---|---|---|---|---|---|---|
| 3 | F2_Area | 34cM | 12.06 | 4.49 | 15 | 4.55 | 1.09 | 18.27 |
| 8 | F2_Area | 9cM | 11.94 | 4.47 | 15 | 5.54 | -0.42 | 24.13 |
| | | | | | | | | ***42.4*** |
| 4 | F2_Breadth | 29cM | 11.41 | 4.3 | 3.71 | 0.61 | -0.26 | 22.19 |
| 1 | F2_Length | 24cM | 8.36 | 3.24 | 7.12 | 0.01 | -1.18 | 10.82 |
| 3 | F2_Length | 35cM | 12.98 | 4.81 | 7.12 | 1.27 | 0.4 | 21.65 |
| 8 | F2_Length | 11cM | 8.92 | 3.44 | 7.26 | 1.03 | -0.12 | 12.76 |
| | | | | | | | | ***45.23*** |
| 8 | F3_Area | 11cM | 7.73 | 3.03 | 661.88 | 220.48 | 85.31 | 15.75 |

**Table 3.5.2 (Cont.)**

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 8 | F3_Breadth | 11cM | 12.64 | 4.69 | 22.21 | 4.35 | 1.7 | 24.43 |
| 1 | F3_Length | 22cM | 8.92 | 3.45 | 52.1 | 4.63 | -7.93 | 17.19 |
| 8 | F3_Length | 12cM | 8.89 | 3.43 | 52.02 | 7.83 | 2.42 | 15.08 |
| | | | | | | | | ***32.27*** |
| 1 | F3_Perimeter | 23cM | 7.49 | 2.94 | 134.64 | 12.46 | -16.62 | 12.44 |
| 8 | F3_Perimeter | 11cM | 10.26 | 3.91 | 135.97 | 23.59 | 8.58 | 19.76 |
| | | | | | | | | ***32.2*** |

## Correlation between Combined F2 and F3 Leaf Traits

A combined F2 and F3 population from both Germany and Edinburgh was generated in order to detect genes responsible for differences between two generations and a number of different leaf traits were recorded for analysis. The correlation between traits was analysed, with the view that traits under the same genetic or environmental control would be correlated. The correlations were estimated using the methods described in Materials and Methods.

Correlations were estimated for leaf traits within and between different populations, in order to find out if traits are affected by the same factors. For F2 leaf area no significantly high correlation was found with F3 leaf area, suggesting that different genes might have affected this trait under different environmental conditions. F2 leaf perimeter showed high correlation with both leaf length and breadth in F2 population, as well as leaf area in the F3 population. However, leaf perimeter did not correlate between different generations. F2 leaf length was correlated with F2 leaf breadth, but not with leaf area or perimeter in the F2 or other traits in the F3 generation. F2 leaf breadth correlated with F3 leaf area, suggesting that they are controlled by the same genes. F3 perimeter was correlated with both F3 leaf length and breadth, suggesting that perimeter is enlarged by both length and breadth, under control of the same genes (Figure 3.5).

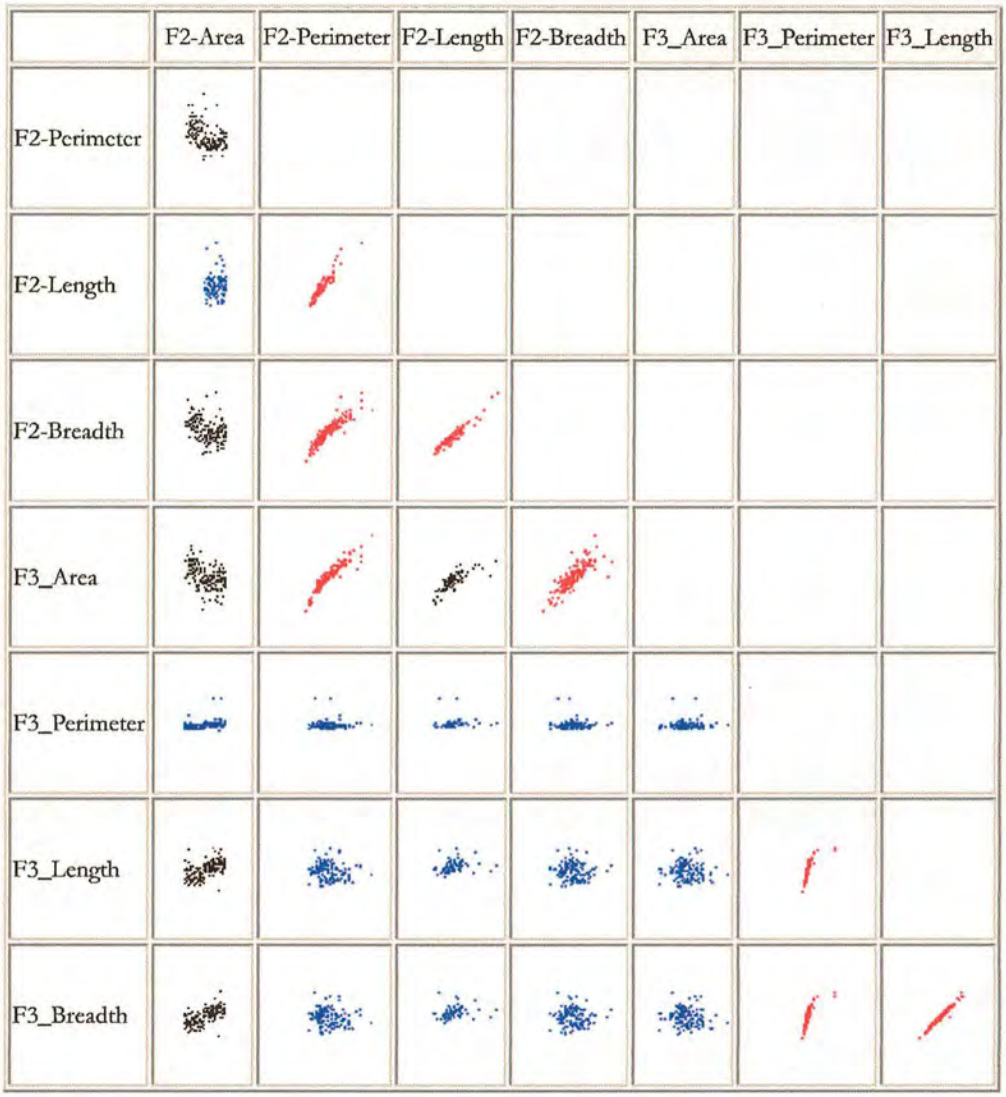



**Figure 3.5 Scatter plots of leaf traits in F2 individuals and the means of their F3 progeny. Red:** high correlation $(r \geq 0.6)$, **black:** intermiddle correlation $(0.3 \leq r < 0.6)$, **blue:** no correlation $(r < 0.3)$.

## QTL mapping With Edinburgh-Germany Combined Map

For QTL mapping with the Edinburgh-Germany combined map, both F2 populations were mapped separately, because they were grown under different environments. Combining the results was expected to detect significant QTLs that were common to both populations. The F3 population were also used in QTL analysis with the combined map using the mean value from each line as an estimate of the value of the F2 parent (n=203). In linkage group 1, QTL for leaf perimeter, maximum length and breadth were mapped to the same region, suggesting that they are controlled by the same

gene. QTL for leaf area, maximum length and breadth were found at the same region in Lg3 and Lg8 of the German F2 population, suggesting that they were controlled by the same genes. QTL for leaf maximum breadth were detected for the German F2 and combined F3 populations, but not for the Edinburgh F2 (Table 3.5.3).

**Table 3.5.3 Results of QTL mapping of F2 from both populations mapped with Edinburgh-Germany combined map.** **G**: Germany line, **E**: Edinburgh line.

| LINKAGE GROUP | TRAIT* | QTL LOCATION | F | LOD | Mean | Additive Effect | Dominance Effect | PVE |
|---|---|---|---|---|---|---|---|---|
| 1 | F3_Length | 0cM | 7.91 | 3.27 | 76.04 | 10.73 | 3.60 | 6.75 |
| 1 | F3_Area | 21cM | 8.60 | 3.55 | 1667.47 | -835.22 | -920.63 | 8.63 |
| 1 | F3_Breadth | 42cM | 12.74 | 5.13 | 35.48 | -5.73 | -8.85 | 9.05 |
| 1 | F3_Perimeter | 44cM | 15.02 | 5.98 | 192.52 | -20.58 | -42.26 | 11.26 |
| 1 | F3_Length | 45cM | 15.91 | 6.30 | 69.07 | -19.74 | -12.70 | 22.30 |
| 3 | F2**E**_Breadth | 4cM | 7.75 | 3.12 | 25.90 | 1.64 | 4.55 | 9.81 |
| 3 | F2**G**_Breadth | 14cM | 13.26 | 4.87 | 35.77 | 7.65 | 4.85 | 21.10 |
| 3 | F2**G**_Area | 15cM | 12.67 | 4.69 | 1607.55 | 482.95 | 109.58 | 19.23 |
| 3 | F2**G**_Length | 16cM | 12.57 | 4.67 | 67.60 | 13.16 | 6.33 | 22.82 |
| 4 | F3_Breadth | 10cM | 7.76 | 3.21 | 35.59 | 3.87 | -5.61 | 6.26 |
| 5 | F2**G**_Breadth | 14cM | 8.39 | 3.25 | 35.77 | -4.03 | -2.99 | 10.29 |
| 7 | F2**E**_Breadth | 37cM | 7.41 | 2.99 | 25.62 | 1.80 | 2.46 | 8.08 |
| 8 | F2**G**_Area | 0cM | 11.44 | 4.31 | 1668.42 | 557.20 | -282.12 | 23.26 |
| 8 | F2**G**_Length | 0cM | 12.57 | 4.66 | 66.55 | 11.40 | -4.89 | 18.67 |
| 8 | F3_Perimeter | 12cM | 8.74 | 3.60 | 200.50 | 27.66 | -6.59 | 7.56 |
| 8 | F2**G**_Breadth | 13cM | 11.04 | 4.17 | 35.77 | 7.42 | -2.88 | 26.97 |
| 8 | F3_Breadth | 13cM | 9.33 | 3.83 | 35.58 | 4.70 | -0.31 | 7.23 |
| 8 | F2**E**_Breadth | 20cM | 14.36 | 5.48 | 25.59 | 3.51 | 0.65 | 24.99 |

## 3.6 Identifying Candidate Hair (Trichomes) Genes

### Identifying candidate hair genes

QTLs underlying the density of different hair types were mapped in the Edinburgh F2 population. In particular, a strong, dominant suppressor of hair formation was identified in *A. majus* on LG6.

Several lines of evidence have implicated a family of *MIXTA*-like genes in specifying hair formation in *Antirrhinum*. *MIXTA* encodes a MYB-related transcription factor that is required for the formation of conical cells in the petal epidermis of *A. majus* (Glover *et al.*, 1998). *MIXTA* can induce both ectopic conical cells and ectopic hairs when mis-expressed in tobacco or in *A. majus* (Glover *et al.*, 2000), suggesting that conical epidermal cells and trichomes might share part of a common developmental pathway that is regulated by *MIXTA-like* (*ML*) genes. Further evidence comes from the finding that *ML1* is expressed in developing hair cells and a subset of conical cells in the *A. majus* flower and is able to induce ectopic hair formation when mis-expressed in the tobacco carpel (Perez-Rodriguez *et al.*, 2005).

*ML2* had been mapped to LG6 close to the dominant suppressor of hair formation in *A. majus*. This raised the possibility that the *ML2* allele from *A. majus* might suppress hairs (e.g. by interfering with other *ML* genes that promote hair formation.)

### Mapping of the MIXTA-like genes

Most F2 plants in the *A. majus* x *A. molle* F2 mapping population were either homozygous for the *A. majus ML2* allele or heterozygous. This is because *ML2* is closely linked to the self-incompatibility (*S*) locus on LG6. Pollen carrying the *S* allele from *A. molle* is unable to grow through the style of the F1 plant, which carries the same *S* allele, and therefore F2 seeds homozygous for the *A. molle S* allele are not produced. Similarly, F2 progeny that are homozygous for the *A. molle ML2* allele cannot occur unless recombination has occurred between *S* and *ML2*, so that the *A. molle ML2* allele is on the same chromosome as the non-functional *A. majus S* allele or because self-incompatibility has broken down.

When F3 progeny of the F2 mapping population were grown, two very hairy plants were found. One of these, No.41, was found to be homozygous for the *A. molle ML2* allele. It was therefore consistent with recombination between the *S*-locus and the gene suppressing hair formation and with *ML2* being the hair density QTL (i.e. the plant was homozygous for the *A. molle* hair allele). Plant No. 41 was also homozygous for the *A. molle* allele of *CYCLOIDEA* (*CYC*) which is linked to *S* and *ML2* on Lg 6, suggesting that the recombination event occurred between the *S*-locus and a region containing *CYC*,*ML2* and the hair locus. To test this further, plant No. 41 was back-crossed to JI98. The F1 progeny were all heterozygous for *ML2*, as expected, and showed a low density of hairs, as in the original F1 of JI98 x *A. molle*. These F1 plants were self-pollinated to produce a F2 family. The F2 showed segregation for hair density and all hairy individuals that were tested were found to be homozygote for the *A. molle ML2* allele. This was consistent with *ML2* being the hair density QTL. However, it did not rule out the possibility that linkage of the hair density QTL to *ML2* was close enough to recover no recombinants in the plants tested.

The other hairy F3 plant, No. 65, was found to be heterozygous for *ML2* but to have a very hairy phenotype similar to No. 41. This suggests either there had been a mutation in the hair inhibitor allele from *A. majus*, in which case *ML2* could still be the hair gene, or there had been recombination between the hair gene and *ML2* and therefore that *ML2* was not the hair gene. The selfed progeny of No. 65 were all hairy, though they segregated for *ML2*, further supporting these views. In a backcross of No.65 x JI98, the F1 population showed similar hair densities but different forms; about 50% had short-glandular trichomes around the basal internodes (~3 to 4 nodes above the cotyledons). Another 50% has long aglandular trichomes around the base of the nodes. In the F2 generation of the backcross, hairiness did not co-segregate with the *ML2* gene. This suggests that *ML2* is not the hairiness gene.

In the F2 of *A. majus* x *A. molle*, plant No 84 was homozygote for the molle *ML2* allele, but was not hairy in phenotype, and its selfed F3 progeny were mostly not hairy, this also suggests that *ML2* is not the hair density QTL.

# Chapter 4: DISCUSSION

The main aim of this research was to investigate genes that underlie the evolutionary differences of *Antirrhinum* species. Most biological traits are genetically complex. Mapping QTL that determine these phenotypes is a powerful way to estimate variation of the genetic architecture for a trait and potentially identify the genes responsible for natural variation. More QTL from reproductive morphs were detected than vegetative morphs (Table 4). An average of two QTL was detected for each floral trait, explaining up to 76% of total variance. Five QTL were found responsible for anthocyanin expression in flowers, explaining up to 62% of the total variance.

**Table 4 Summary of the morphological QTL been found in different traits and its explain percentage.**

| | No. of QTL | PVE | Total explain (%) |
|---|---|---|---|
| Total Length | 3 | 18-27 | 67 % |
| Branching | 3 | 7-47 | 68 % |
| Flowering Time | 1 | 36 | 36 % |
| DL# VLA | 3 | 10-70 | 99 % |
| DL# totA | 2 | 25-75 | 98 % |
| DL# totG | 1 | 51 | 51 % |
| F1 | 2 | 15-20 | 36 % |
| F2 | 2 | 15-25 | 41 % |
| F4 | 2 | 13-15 | 29 % |
| F5 | 3 | 15-32 | 64 % |
| F6 | 3 | 11-33 | 66 % |
| F7 | 2 | 19-25 | 44 % |
| F8 | 4 | 12-24 | 76 % |
| F9 | 1 | 22 | 22 % |
| F11 | 2 | 13-24 | 37 % |
| Flowering Node | 2 | 10-44 | 54 % |
| Leaf Breadth | 2 | 14-17 | 30 % |
| Node Diameter | 2 | 18-20 | 38 % |
| Petal Breadth | 2 | 16-28 | 44 % |
| Red | 2 | 10-49 | 59 % |
| Anthocyanin | 3 | 15-26 | 62 % |
| Yellow | 1 | 31 | 31 % |
| Adax-Leaf-Cell Area | 3 | 27-31 | 87 % |
| Adax-Leaf-Elongation | 3 | 15-25 | 57 % |
| Adax-Leaf-Length | 2 | 13-30 | 44 % |

The genus *Antirrhinum* has ~20 different species distributed in southern Europe along the Mediterranean. Most morphological differences between *Antirrhinum* are quantitative. *A. majus* and *A. molle* were used in this case because they differ in a large number of traits and have already been used to create a recombination map.

## QTL Mapping with Small F2 Population

*A. molle* is naturally outbreeding due to gametophytic self-incompatibility, however, *A. majus* (JI98) has been selfed for more than 10 generations and most of the alleles tested were homozygous. The first *Antirrhinum* genetic map was constructed with mainly RFLP markers (Schwarz-Sommer *et al.*, 2003); however, the previous mapping population had not been scored for many phenotypic data (only three leaf traits had been recorded). Thus, another F2 population was produced from the same parental cross, a new genetic map was constructed and more than 100 traits scored for QTL analysis. AFLP markers were found to provide a low information content for map construction and other types of polymorphic markers, like CAPS and microsatellites were used with AFLP to construct a medium-density genetic linkage map. Such a map can increase the power of detecting QTL (Jung, Fan and Jin, 2005; Kema *et al.*, 2002; Zou, Yandell, and Fine, 2001). Such an approach has been used successfully in Solanaceae, including tomato, potato and eggplant (*Solanum melongena*) to detect QTLs responsible for genome evolution (Doganlar *et al.*, 2002). The power of QTL analysis is, however, also affected by the number of individuals in the mapping population. With a small F2 mapping population (n=107) from the Edinburgh line, the perspicacity of detecting QTL can be reduced; therefore a larger F3 population was generated to increase the ability to detect QTL. The hypothesis was that even after recombinations and segregation in the larger F3 population, the mean of each line would still provide the basis to detect major QTLs. Although some QTL were found in both the F2 and F3 populations, some were found to be different in the two populations and the F3 analysis had a poorer resolution of detection than the F2. In general, the heritability values, $h^2$, for traits, determined by the slope of regression of F3 trait values against parental F2 values (Plomin 1990; Freeman and Heron, 1998) were not high (Table 4.1). This might reflect the different environments under which the two populations were grown; the F2 was grown in a glasshouse with heating and

supplemental lighting while the F3 was grown in an unheated glasshouse in the summer with only natural light. Segregation of non-additive QTL effects in F3 families might also have affected $h^2$ values. For example a recessive QTL allele that was heterozgyous in the F2 would become homozygous in 25% of the F3 and therefore the mean value for the F3 population would be different to the F2 value.

**Table 4.1 Heritability ($h^2$) for leaf traits in the F3 population.**

| F3 heritability $h^2$(%) | Area | Perimeter | Max. Length | Max. Breadth |
|---|---|---|---|---|
| **Edinburgh** | 47.2 | 8.4 | 1.3 | 1.3 |
| **Germany** | 27.4 | - | 27.3 | 40.3 |

## Correlation of Traits

My analysis detected correlations within reproductive or vegetative traits, as well as between them. Traits that are highly correlated are tend to be controlled by the same genes or affected by the same environmental conditions. Floral traits are highly correlated except pedicle length (F1) and the central petal highness (F8). All the upper petal traits are highly correlated with leaf maximum breadth (L-MA-B). Other leaf traits are correlated together with the lower lip width (F7). In epidermal structures, adaxial leaf cell traits are highly correlated (Figure 4).

and However most of the QTL that were detected did not have the same effect on reproductive and vegetative traits. A previous hypothesis is that genes acting pleiotropically on vegetative and floral morphology are likely to limit the ability to evolve, because the vegetative and floral parts of the plant are subjected to different selection pressures. In contrast, species with specialised pollinators, like Antirrhinum, should show independent genetic control of flower traits and leaf traits because these have escaped the effects of pleiotropy, and should show less correlation between floral and vegetative traits. (Berg, 1960; Wolf and Crstolic, 1999). In the case of Antirrhinum, the independent genetic control of leaf and floral traits suggested by QTL analysis supports this

hypothesis. The correlation between floral and vegetative traits detected in F2 mapping populations could therefore reflect linkage of different genes. Alternatively, the power of the QTL analysis might have failed to detect genes acting pleiotropically in leaves and flowers.

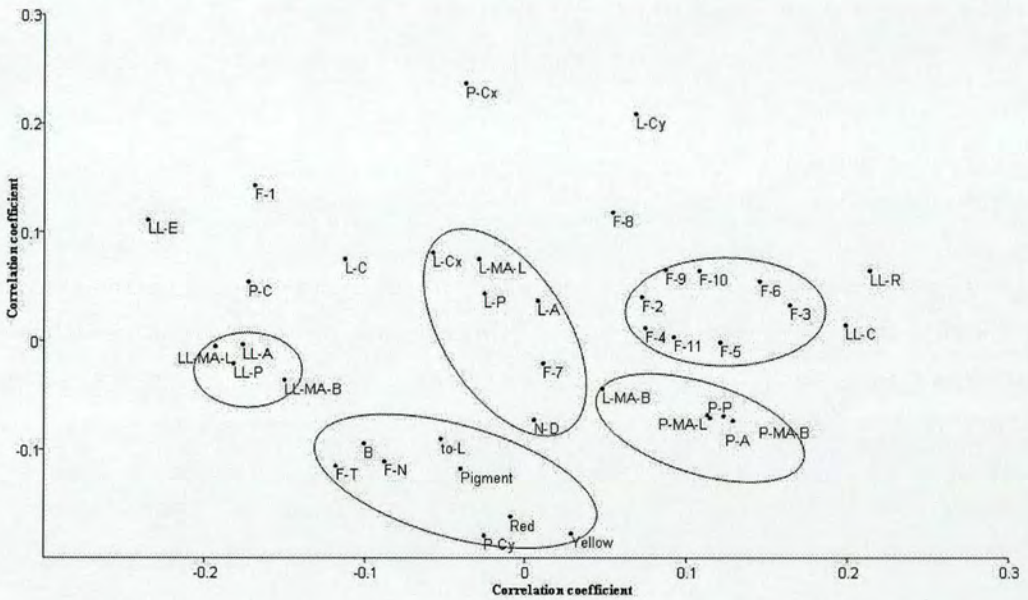



**Figure 4 Correlations between all vegetative and reproductive traits graphically displayed using multidimensional scaling analysis.** The distance between traits is inversely proportional to the size of the correlation coefficient; thus, strongly related traits tend to cluster.

## Epistasis between QTL and the Direction of QTL Effect Shows Traits under Natural Selection or Genetic Drift

The direction of QTL effects can be used to understand whether traits have been under strong natural selection (Orr, 1998). The theory is that strong directional selection (e.g. for differences in leaf size due to water use efficiency) should lead to one species becoming fixed for alleles that increase size - i.e. all the QTL should act in the same direction. On the other hand, if the trait is not selected - i.e. differences have evolved by chance ('under neutrality') then there is likely to be a mixture of increasing and decreasing alleles in the same species. Almost all traits in the F2 population had both positive and negative effects for QTL, suggesting that there has not been a lot of directional selection during the speciation of *A. majus* and *A. molle*.

## Correspondence of QTL to known mutant loci in Antirrhinum

Two different strong QTLs were examined corresponding to known mutant loci in Antirrhinum. One strong QTL for anthocyanin expression in Antirrhinum petal was mapped to the *ROSEA* locus. The other QTL, for trichome density, mapped in the same region as a *MIXTA*-like gene that has been implicated in trichome development.

## • *ROSEA* and *VENOSA* are Involved in Evolution of Flower Pigmentation in *Antirrhinum majus*

A small family of MYB-related proteins are controlling pattern and intensity of flower pigmentation in *Antirrhinum majus*. Those proteins are known to be structurally close to other MYB proteins that regulate anthocyanin biosynthesis in other species such as maize, *Petunia*, tomato and *Arabidopsis* (Cone *et al.*, 1993; Quattrocchio *et al.*, 1999; De Jong *et al.*, 2004; Borevitz *et al.*, 2000). Although, *ROSEA* and *VENOSA* are probably not functionally equivalent to those proteins in other species (Schwinn *et al.*, 2006); Schwinn *et al.* (2006) suggest that MYB proteins have distinct biochemical specificity in terms of their ability to activate transcription from different target promoters. This might reflect differences in their DNA binding affinities and conclude that these

regulatory proteins have diverged functionally as well as in their expression patterns. In the case of *A. majus* and *A. molle*, major QTL for flower pigmentation map to chromosomal regions containing *ROSEA* and *VENOSA*, suggesting that these loci underlie interspecific variation in flower colour.

## • MIXTA-like Proteins are Involved in Trichome Differentiation in Antirrhinum

Trichomes are specified from epidermal precursor cells and can have a number of different functions including protecting plants from herbivores, maintaining temperature or helping seed dispersal as with the fiber-hair in cotton capsules. The MIXTA protein has been found to induce development of both conical cells and trichomes in tobacco (Glover, 2000), but only conical petal cells in its species of origin, *Antirrhinum majus* (Perez-Rodriguez et al., 2005), suggesting that there are other Mixta-like proteins controlling trichome formation in *Antirrhinum*. *Arabidopsis* trichome development has been studied intensively, however, none of the genes known to be involved in trichome development in *Arabidopsis* can be detected in *Antirrhinum*, suggesting that the multi-cellular trichomes in *Antirrhinum* might have a different evolutionary and developmental origin from the unicellular trichomes of *Arabidopsis*.

The *AmMYBML1* gene encodes a protein with similar structure to MIXTA and the ectopic expression of *AmMYBML1* in tobacco has been found to result in conical cell and trichome formation (Perez-Rodriguez *et al.*, 2005). *AmMYBML1* expression was found to correlate with hair and conical cell formation in *A. majus* flowers (Glover *et al.*, 1998). QTL analysis suggested that *A. majus* carries a dominant inhibitor of trichome formation in leaves and stems linked to the *s*-locus in chromosome 6. Because the *AmMYBML2* gene also mapped to this region, it was a candidate for the inhibitor locus. For example, it might act as a dominant negative allele to prevent the function of a related *MIXTA*-like from specifying hairs. However, in one case the trichome inhibitor locus was separated by recombination from the *AmMYBL2* allele of *A. majus*, suggesting that that *AmMYBL2* was not responsible for natural variation in the density of trichomes.

## Evolutionary Implications in Organ Size and Shape Differences

Leaves and floral organs are homologous and share common genetic control of development. Studies of mutants suggest many genes regulate development of both organ types. *graminifolia* and *phantastica* mutants in Antirrhinum, for example, affect development of leaves and petal lobes in the same way (Golz *et al.*, 2004; Waites and Hudson, 1995). Similarly in *Arabidopsis*, the *aintegumenta* gene affects the shape or size of both leaves and petals (Elliott *et al.*, 1996).

One possible adaptive significance of leaf shape and size is that smaller leaves retain a smaller boundary layer of still air (Parkhurst and Louks, 1972). They can therefore lose more heat by convection than larger leaves, which have to transpire more water to maintain the same temperature in sunlight. This predicts that smaller leaves will be advantageous in drier, sunnier habitats. Such a negative correlation between leaf size and water availability has been noted in Australian trees (McDonald *et al.*, 2003).

*Antirrhinum molle*, which has smaller leaves than *A. majus* is native to the foothills of the Pyrenees where it grows on rock faces and screes while *A. majus* is likely to have been domesticated from *A. majus* ssp *pseudomajus* which is more widespread in NE Spain and SW France and grows in deeper soils. Therefore *A. molle* might be adapted to lower water availability (Parkhurst and Louks, 1972). However, *A. molle* has leaves which are densely covered in long hairs. Such hairs are common in alpine plants and have been suggested to protect against overheating in sunlight or against damage by UV light, but in many species appear to have little effect on light absorption, suggesting that their main benefit might be to increase the leaf boundary layer to reduce heat or water loss (Gauslaa, 1984). Therefore the effect of hairs on the boundary layer in *A. molle* might cancel any effects of smaller leaf size.

The adaptive significance of flower shape and size is associated with co-evolution with pollinators. Antirrhinum species are pollinated mainly by bees that are attracted to the face of the flower and use the floral face as a landing platform. Only specific pollinators can land on the flower face and open the flower to enter tube and reach the nectar at its base, picking up pollen in the process. A change in flower shape or size could potentially lead to pollination by another insect and therefore to reproductive isolation. In Aquilegia, for example, evolution of the petal

spur is proposed to have allowed rapid sympatric speciation (Hodges and Arnold, 1995) and altering the length of the spur can change pollinator preference (Fulton and Hodges, 1999). However the relationship between flower and pollinator can be more complex. Some species also differ in colour patterns in the flower to mark the landing position for pollinators. A study of wild radish (*Raphanus sativus*) shows that different flower colours attract different pollinators and also different herbivores. Colour morphs lacking anthocyanin (yellow and white flowers) are a better food source for slugs, while anthocyanin producing morphs (pink and bronze flowers) are more attractive to aphid, thrip and specialist Lepidoptera herbivores. This suggests that differential preference and performance of herbivores for flower colour may cause counter-selection on flower colour exerted by pollinators (Irwin *et al.*, 2005).

## Conservation of Morphological QTL in related species

Major morphological changes, such as change in organ shape and size, have been considered as the result of adaptation over a long term of evolution by selection. Populations attain various genetic differences during the speciation process to become reproductively isolated (Mayr, 1963) and genetic changes causing reproductive isolation are assumed to have a major role in speciation (Maynard, 1983; Macnair and Christie, 1983; Coyne, 1992).

Investigations into the genetic basis of species differences in other taxa have provided different views of the process and tempo of phenotypic divergence. While no previous studies regarding the quantitative genetics of morphology in Antirrhinum, a related species, *Mimulus* (Scrophulariaceae) has been studied. Studies of *Mimulus* have suggested that genes of large effect can contribute to speciation (Bradshaw *et al.*, 1998, 2002). Bradshaw *et al.*, (1998) studied two different *Mimulus* species with conspicuous differences in floral morphology found to be responsible for different pollinators. Large populations were analyzed for 12 different floral traits; an average of eight QTL was found for each trait with each QTL explaining up to 32% of the parental differences (Bradshaw *et al.*, 1998). In order to investigate the genetic basis of reproductive isolating mechanisms in natural populations, Bradshaw *et al.*, (2002) mapped eight floral traits in two sympatric *Mimulus* species that are reproductively isolated owing to pollinator preference by bumblebees

(*M. lewisii*) or hummingbirds (*M. cardinalis*). Relatively few traits of big effect (more than 25%) on floral characters were involved with adaptation to bee or hummingbird pollination. This suggests that speciation had occurred recently and involved strong selection on genes of large effect that contributed to speciation; supporting the previous proposals. In contrast, Fishman *et al.*, (2002) also analyzed two *Mimulus* species with large differences in floral traits; an average of 13 QTL was found for each trait. However, no major (>15% PVE) QTL were detected and none of the traits showed evidence of directional selection. This is similar to the results found for *A. majus* and *A. molle* although more QTL were detected. The result also found that anther and style length are under independent genetic control in Mimulus, suggesting that anther and stigma position can evolve independently, giving rise to self-pollinated species from an outcrossing ancestor. Investigations of the genetic basis for interspecific differences between sunflower (*Helianthus*) species also detected few QTL with low effects in petal length and other floral morphological traits and more genetic variation within each species than between them (Him *et al.*, 1999; Lexer *et al.*, 2005).

The results from analysis of two ecotypes of the same *Silene* species also found a similar number of QTL for floral and leaf traits as other intra-specific studies. Morphological traits that differentiated the two ecotypes were strongly correlated, presumably as a consequence of the joint effects of extensive linkage of QTLs for different traits, pleiotropy and directional selection (Bratteler, Baltisberger and Widmer, 2006). Other intra-species studies in *Arabidopsis* and maize show few QTL and no strong effects and a mixture of positive and negative parental alleles for each trait, providing no evidence of directional selection and consistent with neutral evolution (Westerberg, 2002; El-Lithy, 2004).

## Domestication

In contrast to evolution in the wild, domestication is more likely to have involved strong and artificial selection for agriculturally desirable traits. Although the *A. majus* parent used in QTL analysis was derived from a cultivated variety, it is similar to its wild ancestor, *A. majus* ssp. *pseudomajus*, suggesting that it was not subjected to strong artificial selection during domestication.

Burke *et al.*, (2002) investigated the genetics of sunflower domestication by crossing wild and domesticated sunflowers. They found relatively few QTL with generally low effects (2-10 QTL per trait). Only 4 QTL, involved in seed size, had PVE values greater than 25%. On the basis of the directionality of QTL, strong directional selection for increased seed size appeared to have played the major role in sunflower domestication. No other traits were found with similar evidence of directional selection, and numerous wild alleles with cultivar-like effects were also found. This, combined with the no major QTL, suggests that sunflower was readily domesticated. In most other crops, a small number of QTL with large effects have been detected. In eggplant (aubergine) only 4 QTL increase the fruit weight of a cultivar relative to its wild ancestor with total PVE of 86%, consistent with strong directional selection (Doganlar *et al.*, 2002). Eshed and Zamir (1996) also found a single QTL from *Lycopersicon pimpinellifolium* that decreases fruit yield 40% when introgressed into *L. esculentum*. These finding suggests domestication can involve only a few QTL with small effects or single QTL with strong effect. Some traits under strong direction selection during domestication also show clustering of QTL (Fray *et al.*, 2004; Jiang *et al.*, 1999; Koinange *et al.*, 1996; Cai and Morishima, 2002), which might reflect pleiotropy of individual QTL genes or linkage of different QTL. In the case of Antirrhinum, QTL associated with species differences were not clustered, again suggesting that there was no strong selection during speciation or during domestication of *A. majus*.

Phenotypic differences between species or higher taxa sometimes appear to be non-adaptive (Rieseberg *et al.*, 2002). This could be explained in a number of ways, including neutral evolution, a trait which is subjected to different selection pressures under different conditions (an evolutionary trade-off), or the underlying genes having effects on more than one trait (pleiotropy). Orr (1998) proposed a sign test for detecting QTL effects of genetic drift or natural selection. QTL effects should be mostly in the same direction if the trait has strong directional selection; otherwise QTLs with antagonistic effects should be common (Rieseberg *et al.*, 2002). Domestication traits in general show more evidence of directional selection than non-domestication traits. Differences between species are more likely to involve direction selection that differences within species, suggesting that directional selection is important in speciation. Life history traits (i.e. those affecting

fitness directly) are generally subjected to more directional selection than morphology with selection on physiological traits the lowest.

## From QTL to genes

Once QTL have been confirmed, they can be characterized further in different environmental conditions and genetic backgrounds. In Antirrhinum, the development of the nearly isogenic lines (NILs) approaches can be used to fine map QTL. NILs carrying a single genomic segment which contains the QTL in an otherwise uniform genetic background enable more accurate estimates of the number of QTLs that affect a trait. They are also ideal material for precise estimation of QTL effects and map based gene isolation. This has been used successfully in rice, tomato and *Mimulus* (Zhang et al., 2006; Alpert and Tanksley; Fishman and Willis, 2005).

In some cases, such as in tomato, the QTL can be mapped directly to the gene (Fridman, Pleban and Zamir, 2000; Kroymann et al., 2001). Usually, the selection of candidate genes can begin once QTL have been localized to a relatively narrow region (~3 cM or less). A full genome sequence can also suggest genes in the QTL interval. Once the candidate genes have been identified, there are several ways to test the gene function, such as identification of a null mutation. More than one null allele can often be identified for most candidate genes within the QTL interval, and the quantitative phenotypes of these alleles can be measured later. The final step is to reintroduce alternate alleles into reciprocal QTL lines or null mutant backgrounds to show that each allele has a significantly different effect on the phenotype (El-Din El-Assal et al., 2001;Frary et al.). Another way to confirm a QTL gene is to use gene replacement, which has been demonstrated successfully in rice (Terada et al., 2002). Gene replacement can be used to specifically substitute alleles at the QTL locus while maintaining the correct genomic context, as has been performed in *Drosophila* (Greenberg et al., 2003).

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# APPENDICES

Appendix 1.1 Growth trait scorings

| Plant number | flowering-node | day-to-flower | total-length | branch-no | node-diameter |
|---|---|---|---|---|---|
| 1 | 11 | 83 | 36.2 | 16 | 2.55 |
| 2 | 10 | 72 | 33.7 | 19 | 2.73 |
| 3 | 10 | 76 | 37.1 | 20 | 2.88 |
| 4 | - | - | - | - | 2.77 |
| 5 | 11 | 75 | 34.5 | 19 | 2.28 |
| 6 | - | - | - | - | 2.25 |
| 7 | 6 | 73 | 23.1 | 9 | 2.2 |
| 8 | 8 | 73 | 19.4 | 8 | 2.13 |
| 9 | 12 | 92 | 39 | 16 | 2.07 |
| 10 | 10 | 75 | 27.3 | 15 | 2.17 |
| 11 | 26 | 92 | 45.2 | 28 | 3.03 |
| 12 | - | - | - | - | 2.33 |
| 13 | 7 | 72 | 25.9 | 10 | 2.1 |
| 14 | - | - | - | - | 1.97 |
| 15 | 9 | 73 | 34.7 | 15 | 2.98 |
| 16 | 14 | 86 | 43.5 | 22 | 2.92 |
| 17 | 9 | 72 | 30.9 | 17 | 2.68 |
| 18 | - | - | - | - | 2.63 |
| 19 | 10 | 86 | 38.6 | 17 | 2.57 |
| 20 | 13 | 76 | 41.5 | 23 | 3.02 |
| 21 | - | - | - | - | - |
| 22 | - | - | - | - | - |
| 23 | 8 | 83 | 29.9 | 11 | 2.53 |
| 24 | 9 | 72 | 34.4 | 16 | 2.9 |
| 25 | 13 | 82 | 34.1 | 19 | 2.47 |
| 26 | - | - | - | - | 2.2 |
| 27 | 8 | 72 | 30.6 | 16 | 3 |
| 28 | 16 | 76 | 33.9 | 24 | 2.82 |
| 29 | - | - | - | - | 2.17 |
| 30 | - | - | - | - | 2.13 |
| 31 | 13 | 72 | 37.3 | 19 | 3.13 |
| 32 | - | 89 | - | - | 3.25 |
| 33 | 13 | 73 | 30.3 | 16 | 2.83 |

| | | | | | |
|---|---|---|---|---|---|
| **34** | 12 | 79 | 39.9 | 22 | 2.48 |
| **35** | 10 | 85 | 30.8 | 14 | 2.2 |
| **36** | 13 | 76 | 33.1 | 20 | 3.02 |
| **37** | - | - | - | - | 2.52 |
| **38** | 9 | 79 | 30.7 | 12 | 2.23 |
| **39** | 9 | 85 | 35.4 | 14 | 2.07 |
| **40** | 14 | 79 | 35.5 | 24 | 2.98 |
| **41** | 0 | 72 | 23.7 | 14 | 1.82 |
| **42** | 9 | 72 | 27.1 | 17 | 2.88 |
| **43** | 14 | 78 | 46.5 | 24 | 3.1 |
| **44** | - | - | - | - | - |
| **45** | 9 | 75 | 31.9 | 12 | 2.73 |
| **46** | 10 | 75 | 35.7 | 15 | 2.9 |
| **47** | 10 | 73 | 31.4 | 16 | 2.98 |
| **48** | 11 | 81 | 29 | 19 | 2.75 |
| **49** | 18 | 82 | 42.6 | 23 | 3.53 |
| **50** | 10 | 72 | 30.1 | 18 | 2.57 |
| **51** | 10 | 72 | 29.2 | 19 | 2.23 |
| **52** | 21 | 77 | 43 | 29 | 2.83 |
| **53** | 9 | 77 | 31.7 | 18 | 2.22 |
| **54** | 16 | 78 | 41.8 | 24 | 3.1 |
| **55** | 10 | 79 | 36.3 | 17 | 2.67 |
| **56** | 7 | 72 | 25.1 | 14 | 2.25 |
| **57** | 8 | 72 | 24.4 | 8 | 2.47 |
| **58** | 8 | 72 | 28.1 | 15 | 2.03 |
| **59** | 9 | 82 | 28.2 | 14 | 2.27 |
| **60** | - | - | - | - | 2.48 |
| **61** | 9 | 72 | 31.9 | 15 | 2.38 |
| **62** | 12 | 75 | 35.9 | 20 | 2.6 |
| **63** | 13 | 72 | 35.4 | 19 | 2.68 |
| **64** | 16 | 77 | 41.1 | 25 | 2.6 |
| **65** | - | - | - | - | 2.45 |
| **66** | 11 | 87 | 41.9 | 17 | 2.77 |
| **67** | - | - | - | - | 1.9 |
| **68** | 9 | 75 | 33.7 | 18 | 3.1 |
| **69** | 9 | 72 | 31.1 | 18 | 2.55 |
| **70** | 7 | 81 | 16.9 | 12 | 1.62 |
| **71** | 8 | 75 | 26.6 | 11 | - |

| | | | | | |
|---|---|---|---|---|---|
| **72** | 7 | 72 | 28.4 | 11 | 2.9 |
| **73** | 13 | 81 | 38.9 | 20 | 3.2 |
| **74** | 12 | 78 | 38.2 | 20 | 2.68 |
| **75** | - | - | - | - | - |
| **76** | 12 | 81 | 43.2 | 22 | 2.88 |
| **77** | 8 | 72 | 24.9 | 13 | 2.77 |
| **78** | - | 82 | - | - | 2.9 |
| **79** | 9 | 75 | 36.9 | 17 | 2.73 |
| **80** | 13 | 87 | 46.8 | 18 | 2.8 |
| **81** | - | - | - | - | - |
| **82** | - | - | - | - | 2.2 |
| **83** | 7 | 72 | 24.3 | 13 | 2 |
| **84** | 9 | 73 | 31.9 | 16 | 2.18 |
| **85** | 14 | 89 | 31.7 | 21 | 2.43 |
| **86** | 11 | 89 | 42.9 | 22 | 2.53 |
| **87** | 9 | 81 | 31.7 | 12 | 1.88 |
| **88** | - | - | - | - | - |
| **89** | - | - | - | - | 1.65 |
| **90** | 11 | 76 | 27.5 | 15 | 2.15 |
| **91** | 11 | 76 | 32.1 | 14 | 2.63 |
| **92** | 11 | 88 | 40.8 | 22 | 2.68 |
| **93** | - | - | - | - | 1.9 |
| **94** | - | - | - | - | 2 |
| **95** | 19 | 78 | 44.2 | 25 | 3.42 |
| **96** | - | - | - | - | 2.4 |
| **97** | 17 | 85 | 45.7 | 28 | 3.2 |
| **98** | 9 | 76 | 31.2 | 13 | 2.03 |
| **99** | 11 | 85 | 43.8 | 22 | 2.6 |
| **100** | 12 | 72 | 37.7 | 20 | 2.58 |
| **101** | - | 82 | - | - | 2.42 |
| **102** | 8 | 75 | 33 | 17 | 2.72 |
| **103** | 12 | 77 | 39.3 | 23 | 2.8 |
| **104** | 11 | 76 | 31.5 | 20 | 2.48 |
| **105** | - | - | - | - | 2.5 |
| **106** | 9 | 75 | 34 | 15 | 2.62 |
| **107** | - | - | - | - | 2.13 |

**Appendix 1.2 Flower phenotypes.** **F-1**: pedicel length. **F-2**: flower tube length. **F-3**: flower tube height. **F-4**: mid-lobe width. **F-5**: gibba length. **F-6**: upper petal side length. **F-7**: lower lip width. **F-8**: height from upper petal to lower lip. **F-9**: style length. **F-10**: long anther filament length. **F-11**: short anther filament length.

| Plant number | F-1 | F-2 | F-3 | F-4 | F-5 | F-6 | F-7 | F-8 | F-9 | F-10 | F-11 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 6.1* | 11.5 | 4.7 | 10.6 | 7.9 | 15.3 | 6.5 | 9.8 | 12.6 | 11.8 | 16.5 |
| 2 | 8.4 | 13.2 | 5.4 | 13.8 | 8.7 | 16.7 | 6.9 | 10.8 | 15.4 | 15 | 18 |
| 3 | 7.4 | 12.3 | 4.9 | 11.4 | 7 | 14.8 | 5.4 | 9.5 | 12.8 | 12.5 | 17.2 |
| 4 | - | - | - | - | - | - | - | - | - | - | - |
| 5 | 14 | 11.1 | 3.2 | 10.8 | 6.2 | 13.1 | 5.1 | 8 | 13.4 | 11.6 | 15.1 |
| 6 | - | - | - | - | - | - | - | - | - | - | - |
| 7 | 9.1 | 11.9 | 5.1 | 12.7 | 8.5 | 16.9 | 6.4 | 14.1 | 14 | 13.5 | 17.2 |
| 8 | 7.7 | 11.2 | 2.9 | 10.8 | 6.5 | 15.7 | 5.1 | 12.5 | 14 | 12.5 | 16 |
| 9 | 13.2 | 11.7 | 4.4 | 12.8 | 7.3 | 14.2 | 6 | 12.1 | 15.6 | 12.8 | 17.6 |
| 10 | 5.5 | 12.3 | 5.1 | 14.2 | 8.3 | 15.4 | 6 | 16.3 | 14.3 | 12.5 | 17.4 |
| 11 | 5.1 | 12.7 | 4.4 | 13 | 8.9 | 15.9 | 6 | 12 | 15.2 | 14.9 | 19.5 |
| 12 | - | - | - | - | - | - | - | - | - | - | - |
| 13 | 8.7 | 11.8 | 3.8 | 11.1 | 7.2 | 14.3 | 5.2 | 10.5 | 13.6 | - | 17.6 |
| 14 | - | - | - | - | - | - | - | - | - | - | - |
| 15 | 9.2 | 11 | 4.2 | 10.6 | 7.9 | 13.9 | 4.5 | 11.1 | 13.4 | 13.7 | 17 |
| 16 | 12.8 | 12.3 | 4.2 | 13.5 | 7.4 | 15.6 | 6.9 | 15.1 | 15.5 | 14.1 | 18.5 |
| 17 | 11.1 | 13.8 | 5.6 | 13.7 | 8.9 | 17 | 6.2 | 15 | 15.9 | 15 | 19.3 |
| 18 | - | - | - | - | - | - | - | - | - | - | - |
| 19 | 8.4 | 13.6 | 3.9 | 15.7 | 8.2 | 15.7 | 6.7 | 11.6 | 14.1 | 13.3 | 18 |
| 20 | 7.9 | 14.1 | 5.1 | 13.1 | 9.1 | 14 | 6 | 13.9 | 16 | 14.4 | 19.1 |
| 21 | - | - | - | - | - | - | - | - | - | - | - |
| 22 | - | - | - | - | - | - | - | - | - | - | - |
| 23 | 9.1 | 12.9 | 5.6 | 13.1 | 8.2 | 16 | 6.2 | 11 | 15.1 | 14 | 19 |
| 24 | 7.4 | 13.6 | 7.5 | 12.1 | 8.9 | 16.9 | 5.2 | 13.8 | 13.8 | 13.8 | 18.7 |
| 25 | 14.9 | 12 | 3.9 | 11.5 | 7.4 | 14 | 6.1 | 9.6 | 13.7 | 14.1 | 17.8 |
| 26 | - | - | - | - | - | - | - | - | - | - | - |
| 27 | 8 | 14.6 | 5.4 | 12.4 | 7.8 | 18.7 | 5.5 | 15.7 | 15.7 | 14.4 | 19.6 |
| 28 | 7.5 | 12.3 | 3.3 | 11.1 | 8 | 13.4 | 4 | 11.5 | 13.1 | 13.2 | 17.1 |
| 29 | - | - | - | - | - | - | - | - | - | - | - |
| 30 | - | - | - | - | - | - | - | - | - | - | - |
| 31 | 6.8 | 13.6 | 6 | 13.6 | 9.5 | 16.1 | 4.6 | 9.7 | 16.7 | 13.6 | 18 |
| 32 | 8.7 | 13.2 | 4.3 | 12 | 8.3 | 14 | 6.3 | 10 | 14 | 14.7 | 18.7 |
| 33 | 7.8 | 11.6 | 4.5 | 12.1 | 8.1 | 16.8 | 5.5 | 10.3 | 14.1 | 12.9 | 17.2 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **34** | 8 | 12 | 11.6 | 11 | 7.1 | 12.5 | 4.5 | 8.6 | 14.3 | 13.3 | 17.8 |
| **35** | 6.1 | 11.6 | 3.7 | 11 | 6.6 | 14 | 5.3 | 6.3 | 11.7 | 13 | 16.9 |
| **36** | 4.7 | 13.9 | 4.8 | 14.5 | 8.5 | 15.6 | 7.4 | 12.8 | 14.6 | 13.9 | 18.4 |
| **37** | - | - | - | - | - | - | - | - | - | - | - |
| **38** | 7.9 | 11.8 | 4.2 | 10.6 | 6.5 | 15.9 | 5.8 | 11.4 | 13.5 | 12.8 | 17 |
| **39** | 14.4 | 11.6 | 2.9 | 15.6 | 6.5 | 11.4 | 6.8 | 14 | 15.1 | 12.6 | 16.6 |
| **40** | 9.1 | 12 | 3.7 | 11 | 7.3 | 13.3 | 4.7 | 11.1 | 12.1 | 12.8 | 16.2 |
| **41** | 15.9 | 12.1 | 4.1 | 11.8 | 7.4 | 16.6 | 5 | 13.5 | 14.4 | 13.7 | 16.7 |
| **42** | 5.3 | 13.7 | 4.4 | 12.5 | 9.2 | 15.5 | 7 | 10.8 | 15 | 14 | 17.5 |
| **43** | 8 | 12 | 5 | 12 | 8 | 12 | 5 | 10 | 13 | 13 | 17 |
| **44** | - | - | - | - | - | - | - | - | - | - | - |
| **45** | 6.8 | 12.5 | 4.3 | 13 | 8.8 | 15.6 | 5.2 | 12.4 | 14.4 | 13.6 | 18.6 |
| **46** | 6.4 | 12.1 | 4.9 | 13.2 | 8.9 | 16.8 | 6.2 | 12.8 | 14.6 | 13.2 | 17.3 |
| **47** | 6 | 13.5 | 4.3 | 8.9 | 7.5 | 14.6 | 4.9 | 10.2 | 14.9 | 13.5 | 18.2 |
| **48** | 5.4 | 11.6 | 5.7 | 10 | 7.8 | 17 | 6 | 9.2 | 14 | 13.4 | 17.5 |
| **49** | - | - | - | - | - | - | - | - | - | - | - |
| **50** | 4.1 | 12 | 4.8 | 13.4 | 9 | 15 | 7 | 12.5 | 14.3 | 13.6 | 15.6 |
| **51** | 5.2 | 3.9 | 5.3 | 10.2 | 8 | 12.5 | 6.3 | 13.3 | - | - | - |
| **52** | 10.1 | 11.4 | 4.4 | 10.9 | 7.2 | 12.9 | 5.3 | 9.9 | 13 | 12.5 | 16.4 |
| **53** | 9.4 | 11.4 | 4.1 | 11.2 | 6.5 | 14.7 | 4.6 | 13.9 | 13.3 | 11.8 | 15.8 |
| **54** | 4.8 | 11.8 | 4.8 | 12.5 | 8.7 | 14.1 | 7.1 | 9.9 | 15 | 13.5 | 18.5 |
| **55** | 13.3 | 11 | 3.3 | 10.6 | 7 | 13.2 | 4.3 | 11.8 | 14.1 | 11.8 | 15.8 |
| **56** | 6.5 | 12.2 | 3.8 | 11.6 | 8.2 | 16 | 4.9 | 9.3 | 14.5 | 14.4 | 18 |
| **57** | 6.9 | 13.8 | 6 | 12.9 | 8.2 | 17.9 | 5 | 12.6 | 15.5 | 15.5 | 20.4 |
| **58** | 13.1 | 12.4 | 8 | 11.5 | 8.2 | 15.1 | 5.5 | 15.3 | 14.8 | 13.5 | 17.2 |
| **59** | 9.6 | 12.8 | 5.2 | 12.5 | 7.5 | 15.8 | 4.5 | 9 | 13.9 | 14 | 18.8 |
| **60** | - | - | - | - | - | - | - | - | - | - | - |
| **61** | 11.2 | 14.4 | 5.4 | 14 | 8 | 15.5 | 7.1 | 14 | 14 | 12.8 | 18.1 |
| **62** | 8 | 12 | 4.6 | 12.5 | 8.1 | 16 | 7.1 | 8.4 | 13.7 | 13.1 | 17.5 |
| **63** | 5.6 | 12 | 3.5 | 12.1 | 7.7 | 14.3 | 4.5 | 12.2 | 14.4 | 13.5 | 17.4 |
| **64** | 6.6 | 12.2 | 5 | 13 | 7.7 | 14.1 | 6.8 | 11.4 | 12.4 | 12.4 | 17.4 |
| **65** | 16.9 | 11.7 | 2.9 | 12.1 | 7.4 | 15 | 6.1 | 10.9 | 14 | 12.8 | 16 |
| **66** | 6.2 | 12.4 | 4.6 | 13.1 | 8.1 | 16.7 | 5.5 | 14 | 14.3 | 14 | 18.4 |
| **67** | - | - | - | - | - | - | - | - | - | - | - |
| **68** | 5.8 | 12.5 | 4.2 | 13 | 8.3 | 15.4 | 4.9 | 14 | 13.6 | 13.1 | 17.5 |
| **69** | 10.4 | 12.1 | 3.9 | 11.3 | 7.2 | 15 | 5.7 | 14.3 | 14.8 | 12.9 | 17.3 |
| **70** | 12.7 | 12.4 | 3.1 | 10.7 | 6.5 | 13 | 6.2 | 4.6 | 13 | 13 | 17.2 |
| **71** | 19.4 | 11.8 | 4.5 | 12.9 | 6.9 | 15 | 5.1 | 11 | 14.5 | 16.2 | 12.8 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **72** | 6.5 | 12.8 | 6.2 | 14.8 | 8.4 | 18.9 | 7.1 | 10.5 | 15.5 | 15.6 | 20 |
| **73** | 8.8 | 12.5 | 3.9 | 11.1 | 6.7 | 14 | 4 | 15 | 14.2 | 12.5 | 18.8 |
| **74** | 9.4 | 12.5 | 4.1 | 10.1 | 6.9 | 14.6 | 4.4 | 7.5 | 14.2 | 13.2 | 18.1 |
| **75** | - | - | - | - | - | - | - | - | - | - | - |
| **76** | 9 | 12.3 | 4.6 | 11.5 | 8.1 | 15.1 | 5.9 | 10.4 | 15.2 | 13.3 | 17.8 |
| **77** | 5 | 12.6 | 5.3 | 13 | 8.9 | 17.3 | 6.1 | 11.7 | 15.2 | 15.2 | 19.4 |
| **78** | 7.2 | 13.8 | 5.6 | 13.8 | 8.5 | 15 | 6.5 | 12.9 | 14.8 | 13.9 | 17.4 |
| **79** | 6 | 12.7 | 5.2 | 13.3 | 7.8 | 15.4 | 5.4 | 14.2 | 14.8 | 13 | 17.5 |
| **80** | 6.2 | 11.9 | 4.7 | 13.1 | 8.1 | 15.6 | 6.1 | 10.6 | 13.2 | 13 | 18.4 |
| **81** | - | - | - | - | - | - | - | - | - | - | - |
| **82** | - | - | - | - | - | - | - | - | - | - | - |
| **83** | 11.7 | 13.3 | 4.7 | 13.6 | 8 | 16 | 6.3 | 13.9 | 15.2 | 14.6 | 19.4 |
| **84** | 13.6 | 12.6 | 4.3 | 12.3 | 7.3 | 17.1 | 6.3 | 16 | 16 | 13 | 18.8 |
| **85** | 13.5 | 12.9 | 2.9 | 10.6 | 6.1 | 14.1 | 5 | 11 | 15.6 | 14 | 19 |
| **86** | 15.2 | 13.6 | 5.2 | 14.2 | 7.6 | 17.5 | 6.8 | 17.9 | 16.5 | 14.2 | 19.4 |
| **87** | 8.5 | 12.9 | 3.8 | 12.2 | 7.1 | 14.5 | 6.2 | 13 | 14.8 | 12.5 | 16.6 |
| **88** | - | - | - | - | - | - | - | - | - | - | - |
| **89** | - | - | - | - | - | - | - | - | - | - | - |
| **90** | 2.5 | 11.6 | 4 | 11.7 | 7.8 | 12 | 5 | 10.1 | 11.1 | 11.1 | 15.1 |
| **91** | 9.3 | 13 | 4.9 | 12 | 8.9 | 14 | 5 | 12 | 12.9 | 13.4 | 18.6 |
| **92** | 7 | 13.3 | 5.8 | 13.5 | 8.6 | 17.7 | 7 | 12 | 15.4 | 14 | 19.1 |
| **93** | - | - | - | - | - | - | - | - | - | - | - |
| **94** | - | - | - | - | - | - | - | - | - | - | - |
| **95** | 7.3 | 13 | 6 | 13.4 | 9.1 | 14 | 5.8 | 13 | 14.4 | 14.6 | 18 |
| **96** | - | - | - | - | - | - | - | - | - | - | - |
| **97** | 12.2 | 12.1 | 3.6 | 12.5 | 7.8 | 12.6 | 7 | 11.5 | 13.9 | 12.5 | 16.8 |
| **98** | 8 | 12.6 | 3.3 | 11.8 | 7.6 | 15.9 | 5.1 | 12.9 | 13.2 | 12.5 | 17.7 |
| **99** | 10.7 | 12.5 | 5.3 | 13.7 | 7.4 | 16.4 | 7.4 | 13.8 | 13.6 | 13.1 | 17.9 |
| **100** | 8.7 | 13 | 5.3 | 12.2 | 8.2 | 11.8 | 6 | 13.3 | 16 | 14 | 19.2 |
| **101** | - | - | - | - | - | - | - | - | - | - | - |
| **102** | 9.4 | 13 | 6 | 11 | 8.6 | 15.7 | 4.9 | 11 | 14.1 | 13.6 | 18 |
| **103** | 10.6 | 12.3 | 4.5 | 9.6 | 7.8 | 12.5 | 5.8 | 10 | 13.8 | 13.2 | 17 |
| **104** | 13 | 11.8 | 4.7 | 11.5 | 8.5 | 16.4 | 4.8 | 12.5 | 13 | 14 | 18.5 |
| **105** | - | - | - | - | - | - | - | - | - | - | - |
| **106** | 6.3 | 13 | 6.1 | 13.2 | 8.3 | 18.2 | 6.9 | 15.1 | 14.2 | 13.4 | 18.3 |
| **107** | - | - | - | - | - | - | - | - | - | - | - |

* measurement unit: cm;, missing data: "-"

**Appendix 1.3 Whole leaf phenotypes**

| Plant No | L-Area | L-Perimeter | L-Centroid-x | L-Centroid-y | L-Circularity | L-MA-Length | L-Breadth |
|---|---|---|---|---|---|---|---|
| 1 | 947 | 155 | 127 | 72 | 25 | 47 | 33 |
| 2 | 2761 | 324 | 154 | 94 | 38 | 132 | 37 |
| 3 | 1297 | 170 | 51 | 245 | 22 | 64 | 35 |
| 4 | 858 | 154 | 97 | 52 | 28 | 55 | 29 |
| 5 | 919 | 150 | 148 | 48 | 25 | 57 | 28 |
| 6 | 530 | 106 | 94 | 62 | 21 | 34 | 24 |
| 7 | 823 | 137 | 126 | 259 | 23 | 48 | 29 |
| 8 | 685 | 134 | 134 | 246 | 26 | 50 | 24 |
| 9 | 736 | 137 | 181 | 107 | 26 | 49 | 28 |
| 10 | 1007 | 155 | 97 | 93 | 24 | 57 | 29 |
| 11 | 857 | 150 | 100 | 95 | 26 | 53 | 30 |
| 12 | 671 | 138 | 134 | 102 | 28 | 51 | 24 |
| 13 | 1880 | 281 | 154 | 307 | 42 | 109 | 33 |
| 14 | 615 | 120 | 33 | 74 | 24 | 42 | 25 |
| 15 | 1226 | 184 | 44 | 78 | 28 | 70 | 31 |
| 16 | 1040 | 188 | 40 | 85 | 34 | 61 | 29 |
| 17 | 1193 | 185 | 145 | 146 | 29 | 70 | 30 |
| 18 | 719 | 166 | 78 | 43 | 39 | 59 | 23 |
| 19 | 760 | 145 | 120 | 103 | 28 | 44 | 29 |
| 20 | 1142 | 173 | 148 | 252 | 26 | 69 | 30 |
| 21 | 613 | 128 | 69 | 333 | 27 | 47 | 23 |
| 22 | - | - | - | - | - | - | - |
| 23 | 744 | 140 | 41 | 91 | 26 | 46 | 27 |
| 24 | 1108 | 168 | 138 | 56 | 26 | 63 | 32 |
| 25 | 726 | 136 | 86 | 215 | 25 | 46 | 27 |
| 26 | 374 | 102 | 82 | 226 | 28 | 35 | 18 |
| 27 | 1466 | 200 | 149 | 33 | 27 | 75 | 33 |
| 28 | 946 | 175 | 139 | 166 | 33 | 62 | 29 |
| 29 | 642 | 137 | 160 | 180 | 29 | 51 | 24 |
| 30 | 598 | 125 | 52 | 98 | 26 | 47 | 23 |
| 31 | 1521 | 185 | 46 | 123 | 23 | 64 | 40 |
| 32 | 864 | 158 | 160 | 124 | 29 | 56 | 30 |
| 33 | 872 | 137 | 97 | 257 | 22 | 46 | 31 |
| 34 | 940 | 163 | 50 | 197 | 28 | 55 | 30 |
| 35 | 864 | 144 | 49 | 128 | 24 | 53 | 26 |
| 36 | 1011 | 154 | 70 | 230 | 23 | 51 | 32 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **37** | 1024 | 185 | 178 | 105 | 34 | 62 | 30 |
| **38** | 1033 | 186 | 22 | 37 | 34 | 58 | 31 |
| **39** | 1042 | 183 | 121 | 65 | 32 | 63 | 30 |
| **40** | 1082 | 178 | 133 | 184 | 29 | 65 | 31 |
| **41** | 761 | 134 | 16 | 266 | 24 | 53 | 25 |
| **42** | 874 | 145 | 101 | 257 | 24 | 52 | 28 |
| **43** | 1222 | 181 | 46 | 152 | 27 | 65 | 33 |
| **44** | - | - | - | - | - | - | - |
| **45** | 1101 | 165 | 101 | 173 | 25 | 58 | 33 |
| **46** | 1391 | 174 | 59 | 167 | 22 | 60 | 39 |
| **47** | 1372 | 189 | 101 | 130 | 26 | 71 | 35 |
| **48** | 845 | 135 | 46 | 247 | 22 | 49 | 28 |
| **49** | 1019 | 162 | 96 | 248 | 26 | 55 | 32 |
| **50** | 899 | 149 | 140 | 96 | 25 | 55 | 28 |
| **51** | 738 | 130 | 30 | 263 | 23 | 50 | 25 |
| **52** | 947 | 154 | 68 | 256 | 25 | 59 | 28 |
| **53** | 845 | 157 | 45 | 33 | 29 | 55 | 29 |
| **54** | 1861 | 232 | 149 | 151 | 29 | 82 | 40 |
| **55** | 895 | 162 | 39 | 111 | 30 | 61 | 27 |
| **56** | 473 | 125 | 47 | 237 | 33 | 49 | 18 |
| **57** | 912 | 141 | 39 | 180 | 22 | 53 | 29 |
| **58** | 702 | 133 | 107 | 37 | 25 | 48 | 25 |
| **59** | 812 | 140 | 70 | 138 | 24 | 48 | 29 |
| **60** | 640 | 141 | 51 | 34 | 31 | 51 | 25 |
| **61** | 2881 | 297 | 153 | 485 | 33 | 114 | 42 |
| **62** | 830 | 142 | 129 | 189 | 24 | 49 | 29 |
| **63** | 1084 | 163 | 161 | 245 | 25 | 58 | 33 |
| **64** | 1078 | 161 | 145 | 194 | 24 | 56 | 33 |
| **65** | 894 | 147 | 99 | 98 | 24 | 55 | 29 |
| **66** | 1178 | 187 | 156 | 119 | 30 | 61 | 35 |
| **67** | 450 | 110 | 80 | 251 | 27 | 37 | 20 |
| **68** | 1209 | 181 | 147 | 57 | 27 | 66 | 31 |
| **69** | 825 | 168 | 40 | 118 | 34 | 64 | 25 |
| **70** | 425 | 103 | 118 | 238 | 25 | 42 | 17 |
| **71** | 580 | 127 | 42 | 269 | 28 | 46 | 23 |
| **72** | 1490 | 187 | 48 | 166 | 23 | 69 | 36 |
| **73** | 1721 | 258 | 95 | 142 | 39 | 92 | 38 |
| **74** | 1202 | 186 | 52 | 48 | 29 | 68 | 33 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **75** | - | - | - | - | - | - | - |
| **76** | 1027 | 161 | 96 | 42 | 25 | 61 | 30 |
| **77** | 1234 | 183 | 44 | 194 | 27 | 67 | 31 |
| **78** | 1255 | 179 | 71 | 98 | 26 | 61 | 35 |
| **79** | 916 | 152 | 96 | 213 | 25 | 54 | 29 |
| **80** | 1079 | 171 | 66 | 107 | 27 | 55 | 35 |
| **81** | - | - | - | - | - | - | - |
| **82** | 657 | 118 | 154 | 182 | 21 | 44 | 25 |
| **83** | 1193 | 184 | 140 | 155 | 28 | 68 | 31 |
| **84** | 1622 | 241 | 96 | 70 | 36 | 79 | 40 |
| **85** | 606 | 137 | 172 | 58 | 31 | 47 | 23 |
| **86** | - | 179 | 58 | 111 | 32 | 64 | 29 |
| **87** | 1097 | 165 | 97 | 192 | 25 | 62 | 32 |
| **88** | 1455 | 210 | 155 | 338 | 30 | 78 | 35 |
| **89** | 385 | 103 | 93 | 174 | 27 | 40 | 16 |
| **90** | 579 | 109 | 153 | 127 | 20 | 40 | 23 |
| **91** | 1059 | 147 | 92 | 76 | 20 | 54 | 32 |
| **92** | 768 | 127 | 128 | 106 | 21 | 40 | 30 |
| **93** | 1094 | 178 | 151 | 252 | 29 | 53 | 34 |
| **94** | 734 | 137 | 36 | 155 | 25 | 49 | 27 |
| **95** | 1496 | 227 | 153 | 61 | 35 | 72 | 38 |
| **96** | 507 | 114 | 170 | 39 | 25 | 43 | 20 |
| **97** | 1316 | 186 | 50 | 45 | 26 | 66 | 35 |
| **98** | 1001 | 153 | 79 | 107 | 23 | 54 | 34 |
| **99** | 1001 | 162 | 141 | 137 | 26 | 57 | 32 |
| **100** | 982 | 164 | 111 | 110 | 27 | 61 | 28 |
| **101** | 786 | 154 | 38 | 251 | 30 | 58 | 26 |
| **102** | 1334 | 188 | 50 | 254 | 27 | 70 | 32 |
| **103** | 949 | 156 | 45 | 105 | 26 | 57 | 29 |
| **104** | 920 | 151 | 94 | 40 | 25 | 54 | 30 |
| **105** | 755 | 149 | 139 | 227 | 29 | 50 | 27 |
| **106** | 1508 | 180 | 47 | 33 | 22 | 67 | 35 |
| **107** | 853 | 155 | 161 | 40 | 28 | 55 | 29 |

**Appendix 1.4 Upper petal phenotype**

| | P-Area | P-Perimeter | P-Centroid-x | P-Centroid-y | P-Circularity | P-MajorAxis-Length | P-Breadth |
|---|---|---|---|---|---|---|---|
| **1** | 146 | 56 | 11 | 271 | 21 | 20 | 12 |
| **2** | 235 | 64 | 30 | 263 | 17 | 24 | 15 |
| **3** | 186 | 58 | 49 | 258 | 18 | 22 | 12 |
| **4** | 245 | 69 | 68 | 251 | 20 | 27 | 15 |
| **5** | 132 | 54 | 86 | 248 | 22 | 20 | 10 |
| **6** | 231 | 65 | 103 | 241 | 18 | 24 | 15 |
| **7** | 210 | 63 | 121 | 232 | 19 | 24 | 14 |
| **8** | 148 | 54 | 139 | 225 | 20 | 21 | 11 |
| **9** | 172 | 54 | 156 | 217 | 17 | 20 | 12 |
| **10** | 248 | 68 | 173 | 205 | 19 | 25 | 15 |
| **11** | 238 | 69 | 14 | 181 | 20 | 27 | 14 |
| **12** | 161 | 56 | 32 | 173 | 20 | 21 | 12 |
| **13** | 158 | 55 | 51 | 167 | 19 | 21 | 12 |
| **14** | 205 | 64 | 67 | 159 | 20 | 25 | 13 |
| **15** | 148 | 51 | 82 | 152 | 18 | 20 | 11 |
| **16** | 197 | 62 | 96 | 144 | 20 | 23 | 13 |
| **17** | 180 | 62 | 112 | 134 | 22 | 24 | 12 |
| **18** | 194 | 64 | 128 | 123 | 21 | 25 | 13 |
| **19** | 225 | 69 | 143 | 111 | 21 | 26 | 14 |
| **20** | 190 | 59 | 161 | 103 | 18 | 23 | 14 |
| **21** | 230 | 68 | 14 | 77 | 20 | 26 | 15 |
| **22** | - | - | - | - | - | - | - |
| **23** | 184 | 61 | 33 | 73 | 20 | 23 | 13 |
| **24** | 206 | 60 | 52 | 64 | 17 | 22 | 14 |
| **25** | 147 | 53 | 67 | 57 | 19 | 21 | 11 |
| **26** | 160 | 54 | 83 | 52 | 18 | 20 | 12 |
| **27** | 184 | 59 | 99 | 42 | 19 | 23 | 12 |
| **28** | 133 | 54 | 114 | 36 | 22 | 22 | 11 |
| **29** | 166 | 58 | 128 | 28 | 21 | 23 | 11 |
| **30** | 210 | 70 | 145 | 20 | 24 | 28 | 13 |
| **31** | 213 | 63 | 11 | 266 | 18 | 22 | 15 |
| **32** | 218 | 66 | 27 | 259 | 20 | 24 | 15 |
| **33** | 202 | 65 | 42 | 249 | 21 | 25 | 13 |
| **34** | 191 | 60 | 58 | 244 | 19 | 23 | 13 |
| **35** | 165 | 57 | 75 | 237 | 19 | 21 | 13 |
| **36** | 228 | 64 | 91 | 227 | 18 | 23 | 14 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **37** | 197 | 61 | 109 | 218 | 19 | 23 | 13 |
| **38** | 241 | 66 | 126 | 207 | 18 | 25 | 14 |
| **39** | 178 | 57 | 144 | 201 | 18 | 22 | 12 |
| **40** | 169 | 56 | 163 | 197 | 19 | 21 | 14 |
| **41** | - | - | - | - | - | - | - |
| **42** | 220 | 67 | 16 | 166 | 20 | 25 | 14 |
| **43** | 205 | 59 | 36 | 161 | 17 | 21 | 14 |
| **44** | 285 | 72 | 56 | 154 | 18 | 27 | 17 |
| **45** | 261 | 69 | 76 | 147 | 18 | 26 | 16 |
| **46** | 250 | 69 | 95 | 139 | 19 | 26 | 15 |
| **47** | 208 | 65 | 116 | 133 | 21 | 25 | 14 |
| **48** | 216 | 63 | 135 | 126 | 18 | 25 | 14 |
| **49** | 189 | 58 | 143 | 83 | 18 | 22 | 14 |
| **50** | 185 | 54 | 173 | 111 | 15 | 19 | 14 |
| **51** | 173 | 56 | 18 | 90 | 18 | 21 | 13 |
| **52** | 173 | 60 | 34 | 82 | 21 | 23 | 12 |
| **53** | - | - | - | - | - | - | - |
| **54** | 231 | 67 | 49 | 74 | 20 | 24 | 15 |
| **55** | 132 | 49 | 64 | 71 | 18 | 18 | 11 |
| **56** | 177 | 58 | 82 | 66 | 19 | 21 | 13 |
| **57** | 261 | 67 | 99 | 56 | 17 | 25 | 16 |
| **58** | 161 | 56 | 116 | 52 | 19 | 20 | 13 |
| **59** | - | - | - | - | - | - | - |
| **60** | 216 | 65 | 149 | 41 | 20 | 23 | 15 |
| **61** | 250 | 67 | 15 | 276 | 18 | 25 | 15 |
| **62** | 279 | 71 | 34 | 270 | 18 | 26 | 17 |
| **63** | 210 | 62 | 51 | 265 | 18 | 23 | 14 |
| **64** | 254 | 67 | 69 | 259 | 18 | 25 | 16 |
| **65** | 161 | 56 | 86 | 255 | 20 | 22 | 12 |
| **66** | 215 | 64 | 102 | 247 | 19 | 25 | 14 |
| **67** | 221 | 65 | 121 | 239 | 19 | 25 | 14 |
| **68** | 214 | 64 | 139 | 234 | 19 | 23 | 15 |
| **69** | 175 | 57 | 156 | 228 | 18 | 22 | 12 |
| **70** | 140 | 53 | 172 | 220 | 20 | 21 | 11 |
| **71** | 157 | 54 | 188 | 213 | 18 | 20 | 12 |
| **72** | 288 | 75 | 15 | 200 | 19 | 28 | 16 |
| **73** | 117 | 46 | 31 | 198 | 18 | 15 | 12 |
| **74** | 212 | 66 | 47 | 187 | 21 | 25 | 13 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **75** | - | - | - | - | - | - | - |
| **76** | 232 | 66 | 66 | 179 | 19 | 25 | 15 |
| **77** | 183 | 59 | 84 | 170 | 19 | 22 | 13 |
| **78** | 257 | 68 | 101 | 165 | 18 | 25 | 16 |
| **79** | 233 | 63 | 121 | 158 | 17 | 22 | 15 |
| **80** | 214 | 67 | 137 | 151 | 21 | 25 | 14 |
| **81** | 220 | 66 | 153 | 141 | 20 | 25 | 14 |
| **82** | 216 | 63 | 169 | 133 | 18 | 24 | 14 |
| **83** | 149 | 61 | 13 | 125 | 25 | 24 | 10 |
| **84** | 211 | 62 | 186 | 121 | 19 | 24 | 13 |
| **85** | 298 | 80 | 31 | 116 | 21 | 31 | 16 |
| **86** | 158 | 57 | 47 | 111 | 21 | 21 | 12 |
| **87** | 256 | 73 | 67 | 102 | 21 | 26 | 17 |
| **88** | 133 | 52 | 87 | 99 | 20 | 20 | 11 |
| **89** | 147 | 53 | 103 | 91 | 19 | 18 | 12 |
| **90** | 215 | 64 | 123 | 80 | 19 | 24 | 14 |
| **91** | 263 | 74 | 141 | 65 | 21 | 28 | 15 |
| **92** | 319 | 76 | 159 | 55 | 18 | 28 | 18 |
| **93** | 227 | 68 | 176 | 43 | 20 | 25 | 14 |
| **94** | - | - | - | - | - | - | - |
| **95** | - | - | - | - | - | - | - |
| **96** | 211 | 62 | 192 | 33 | 18 | 23 | 14 |
| **97** | 182 | 58 | 12 | 270 | 18 | 22 | 12 |
| **98** | 241 | 65 | 29 | 266 | 18 | 24 | 15 |
| **99** | 175 | 56 | 46 | 260 | 18 | 21 | 12 |
| **100** | - | - | - | - | - | - | - |
| **101** | 164 | 56 | 61 | 254 | 19 | 22 | 12 |
| **102** | 146 | 52 | 78 | 250 | 19 | 20 | 12 |
| **103** | 245 | 68 | 96 | 240 | 19 | 26 | 14 |
| **104** | 260 | 69 | 113 | 235 | 18 | 26 | 15 |
| **105** | 298 | 73 | 130 | 227 | 18 | 26 | 17 |
| **106** | 157 | 57 | 149 | 222 | 21 | 21 | 12 |
| **107** | - | - | - | - | - | - | - |

**Appendix 1.5 Petal pigment analysis**

| | Red | Yellow | pigment-analysis |
|---|---|---|---|
| 1 | 4 | 2 | 0.414 |
| 2 | 3.5 | 1 | 0.37 |
| 3 | 3.5 | 1.5 | 0.686 |
| 4 | 3.75 | 0.2 | 0.794 |
| 5 | 3.75 | 1 | 0.376 |
| 6 | 3.25 | 1 | 0.994 |
| 7 | 0 | 0.2 | 0.051 |
| 8 | 2.75 | 1.25 | 0.202 |
| 9 | - | - | 0.004 |
| 10 | 3.75 | 2 | 0.61 |
| 11 | 4.25 | 0.75 | 2.121 |
| 12 | 4 | 1 | 1.237 |
| 13 | 3.75 | 0.75 | 0.21 |
| 14 | 1.25 | 2 | 0.454 |
| 15 | 1 | 0.35 | 0.005 |
| 16 | 4 | 2 | 1.002 |
| 17 | 0 | 0.75 | 0.004 |
| 18 | 1 | 1 | - |
| 19 | 4 | 1.5 | 0.743 |
| 20 | 0.25 | 0.5 | 0.021 |
| 21 | - | - | - |
| 22 | - | - | - |
| 23 | 3.25 | 1.25 | 0.488 |
| 24 | 0.75 | 0.2 | 0.851 |
| 25 | 4 | 1 | 0.911 |
| 26 | 4 | 0.2 | 0.616 |
| 27 | 1.5 | 0.5 | 0.116 |
| 28 | 2 | 1 | 0.237 |
| 29 | 1.25 | 0.5 | 0.147 |
| 30 | 4 | 0.2 | 0.828 |
| 31 | 4 | 1.5 | 0.795 |
| 32 | 4.25 | 1 | 1.28 |
| 33 | 4 | 1 | 1.104 |
| 34 | 1 | 1.25 | 0.051 |
| 35 | 4 | - | 0.803 |
| 36 | 4 | 1.5 | 0.458 |

| | | | |
|---|---|---|---|
| **37** | 4 | 1 | 1.005 |
| **38** | 3 | 0.75 | 0.37 |
| **39** | 2.75 | 0.2 | 0.492 |
| **40** | 3.75 | 1.75 | 0.554 |
| **41** | 1.25 | 0.5 | - |
| **42** | 2 | 0.5 | 0.079 |
| **43** | 3.75 | 1.75 | 0.507 |
| **44** | 3.25 | 1 | 0.57 |
| **45** | 3 | 1.5 | 0.25 |
| **46** | 3.75 | 2 | 0.35 |
| **47** | 2.5 | 0.35 | 0.184 |
| **48** | 2.25 | 1 | 0.183 |
| **49** | 2.75 | 1.5 | 0.487 |
| **50** | 4 | 1 | 0.566 |
| **51** | 3.25 | 2 | 0.239 |
| **52** | 0 | 1.25 | 0.025 |
| **53** | 3.5 | 1 | - |
| **54** | 4.5 | 1 | 1.003 |
| **55** | 1 | 0.5 | 0.031 |
| **56** | 4 | 1 | 0.497 |
| **57** | 1.25 | 0.5 | 0.12 |
| **58** | 3.25 | 1 | 0.344 |
| **59** | 3 | 1.25 | 0.297 |
| **60** | 3 | 1.25 | 0.366 |
| **61** | 0 | 1 | 0.006 |
| **62** | 4 | 1 | 0.687 |
| **63** | 3.5 | 1.5 | 0.324 |
| **64** | 3.5 | 1.5 | 0.394 |
| **65** | 0.5 | 0.2 | 0.029 |
| **66** | 2.75 | 0.35 | 0.471 |
| **67** | 4 | 1 | 0.409 |
| **68** | 3.5 | 1 | 0.452 |
| **69** | 0 | 0.35 | - |
| **70** | 3 | 0.6 | 0.358 |
| **71** | 2 | 0.75 | 0.117 |
| **72** | 4 | 1 | 0.35 |
| **73** | 3.75 | 0.6 | 0.97 |
| **74** | 3.75 | 0.5 | 0.566 |

| | | | |
|---|---|---|---|
| **75** | - | - | - |
| **76** | 4 | 1 | 1.115 |
| **77** | 2.25 | 0.35 | 0.302 |
| **78** | 4 | 1 | - |
| **79** | 2.75 | 1 | 0.422 |
| **80** | 3.75 | 2 | 0.911 |
| **81** | - | - | - |
| **82** | 2 | 0.5 | 0.178 |
| **83** | 2.25 | 0.35 | 0.104 |
| **84** | 2.5 | 0.5 | 0.511 |
| **85** | 4.5 | 1 | 2.117 |
| **86** | 4 | 0.2 | 1.01 |
| **87** | 0.75 | 0.75 | 0.038 |
| **88** | - | - | - |
| **89** | 0 | 1.25 | 0.07 |
| **90** | 2.25 | 0.75 | 0.09 |
| **91** | 3 | 1.5 | 0.132 |
| **92** | 1.25 | 1.5 | 0.061 |
| **93** | 3 | 2 | 0.241 |
| **94** | 4 | 0.2 | 0.601 |
| **95** | 3.75 | 1 | - |
| **96** | 0 | 1 | - |
| **97** | 4.25 | 1.5 | 0.833 |
| **98** | 3 | 0.75 | 0.229 |
| **99** | 3.5 | 2 | 0.453 |
| **100** | 4 | 1.5 | 0.781 |
| **101** | - | - | - |
| **102** | 0.75 | 1 | 0.014 |
| **103** | 2.75 | 1 | 0.234 |
| **104** | 0 | 1 | 0.01 |
| **105** | 4.5 | 1.25 | 0.851 |
| **106** | 2.5 | 0.5 | - |
| **107** | 0.75 | 0.2 | 0.013 |

**Appendix 1.6 Adaxial leaf epidemical cell scoring**

| | Area | Perimeter | Max. Length | Max. Breadth | Elongation | Roundness | Compactness |
|---|---|---|---|---|---|---|---|
| **1** | 328.25 | 131.33 | 33.48 | 18.64 | 1.85 | 0.25 | 0.61 |
| **2** | 471.04 | 160.63 | 39.93 | 23.48 | 1.76 | 0.25 | 0.62 |
| **3** | - | - | - | - | - | - | - |
| **4** | - | - | - | - | - | - | - |
| **5** | 427.73 | 149.67 | 39.32 | 20.74 | 1.95 | 0.26 | 0.6 |
| **6** | - | - | - | - | - | - | - |
| **7** | 303.24 | 94.69 | 29.02 | 17.12 | 1.75 | 0.43 | 0.68 |
| **8** | - | - | - | - | - | - | - |
| **9** | 299.72 | 111.4 | 30.27 | 17.52 | 1.77 | 0.33 | 0.65 |
| **10** | 333.87 | 99.83 | 30.41 | 17.94 | 1.74 | 0.44 | 0.68 |
| **11** | 401.52 | 136.41 | 36.69 | 20.47 | 1.8 | 0.29 | 0.62 |
| **12** | - | - | - | - | - | - | - |
| **13** | - | - | - | - | - | - | - |
| **14** | - | - | - | - | - | - | - |
| **15** | 475.71 | 157.92 | 42.07 | 21.98 | 2 | 0.26 | 0.58 |
| **16** | 455.97 | 144.02 | 39.11 | 21.8 | 1.83 | 0.29 | 0.62 |
| **17** | 409.27 | 118.63 | 36.45 | 18.58 | 2 | 0.38 | 0.63 |
| **18** | 369.53 | 141.85 | 36.08 | 20.33 | 1.88 | 0.25 | 0.6 |
| **19** | 284.57 | 96.88 | 29.36 | 16.13 | 1.87 | 0.39 | 0.65 |
| **20** | 303.81 | 100.06 | 29.31 | 17.17 | 1.78 | 0.41 | 0.67 |
| **21** | 284.16 | 86.65 | 29.16 | 15.65 | 1.91 | 0.49 | 0.66 |
| **22** | 377.96 | 127.91 | 36.7 | 19.5 | 1.95 | 0.3 | 0.6 |
| **23** | 271.98 | 90.13 | 26.9 | 17.45 | 1.57 | 0.42 | 0.69 |
| **24** | 237.56 | 81.87 | 25 | 15.45 | 1.67 | 0.46 | 0.7 |
| **25** | 379.94 | 123.38 | 35.75 | 18.92 | 1.97 | 0.33 | 0.61 |
| **26** | - | - | - | - | - | - | - |
| **27** | 292.55 | 97.13 | 28.73 | 16.97 | 1.78 | 0.41 | 0.67 |
| **28** | 424.02 | 127.18 | 36.69 | 20.79 | 1.85 | 0.35 | 0.63 |
| **29** | 616.82 | 171.15 | 46.17 | 25.13 | 1.9 | 0.28 | 0.61 |
| **30** | 343.33 | 93.6 | 31.29 | 17 | 1.91 | 0.51 | 0.68 |
| **31** | 257 | 73.38 | 25.09 | 15.59 | 1.67 | 0.6 | 0.73 |
| **32** | 559.82 | 162.52 | 43.12 | 24.89 | 1.77 | 0.29 | 0.62 |
| **33** | 393.12 | 115.19 | 35.66 | 19.73 | 1.88 | 0.38 | 0.63 |
| **34** | 404.01 | 122.62 | 35.09 | 19.77 | 1.84 | 0.37 | 0.65 |
| **35** | 430.13 | 133.17 | 36.41 | 21.15 | 1.78 | 0.32 | 0.64 |
| **36** | 432.06 | 137.03 | 36.1 | 21.39 | 1.71 | 0.31 | 0.65 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **37** | 477.97 | 147.98 | 41.51 | 21.41 | 1.98 | 0.29 | 0.59 |
| **38** | 301.98 | 111.85 | 30.27 | 17.8 | 1.76 | 0.31 | 0.65 |
| **39** | 479.97 | 149.01 | 39.66 | 22.37 | 1.81 | 0.29 | 0.62 |
| **40** | 437.06 | 152.49 | 39.57 | 22.56 | 1.85 | 0.24 | 0.59 |
| **41** | 403.39 | 123 | 34.19 | 20.6 | 1.71 | 0.35 | 0.67 |
| **42** | 503.13 | 155.66 | 40.94 | 23.27 | 1.82 | 0.27 | 0.62 |
| **43** | 375.42 | 119.14 | 34.33 | 19.36 | 1.84 | 0.34 | 0.64 |
| **44** | 364.99 | 112 | 32.5 | 19.57 | 1.71 | 0.38 | 0.67 |
| **45** | 337.78 | 106.92 | 31.46 | 18.31 | 1.78 | 0.38 | 0.66 |
| **46** | 317.55 | 99.72 | 30.51 | 17.21 | 1.83 | 0.41 | 0.67 |
| **47** | 316.09 | 90.66 | 29.85 | 16.4 | 1.88 | 0.5 | 0.68 |
| **48** | 400.49 | 116.74 | 35.77 | 18.75 | 1.97 | 0.39 | 0.64 |
| **49** | 388.06 | 126.49 | 35.71 | 20.35 | 1.82 | 0.31 | 0.63 |
| **50** | 344.8 | 118.39 | 33.69 | 18.82 | 1.86 | 0.32 | 0.63 |
| **51** | 286.07 | 105.59 | 29.41 | 17.35 | 1.75 | 0.34 | 0.65 |
| **52** | 549.02 | 152.78 | 43.72 | 23.48 | 1.89 | 0.32 | 0.61 |
| **53** | 371.2 | 110.99 | 33.04 | 19.03 | 1.82 | 0.4 | 0.66 |
| **54** | 438.16 | 129.06 | 35.7 | 21.73 | 1.67 | 0.34 | 0.66 |
| **55** | 509.15 | 159.91 | 43.46 | 22.84 | 1.96 | 0.27 | 0.59 |
| **56** | - | - | - | - | - | - | - |
| **57** | 361.81 | 99.35 | 30.38 | 19.43 | 1.6 | 0.47 | 0.71 |
| **58** | 329.87 | 106.82 | 31.65 | 17.97 | 1.78 | 0.37 | 0.65 |
| **59** | 308.75 | 96.3 | 28.25 | 17.9 | 1.64 | 0.42 | 0.69 |
| **60** | 431.34 | 142.53 | 39.36 | 20.53 | 2 | 0.28 | 0.59 |
| **61** | 396.29 | 131.58 | 35.19 | 20.94 | 1.73 | 0.31 | 0.64 |
| **62** | 363.03 | 120.8 | 33.69 | 19.2 | 1.82 | 0.32 | 0.64 |
| **63** | 274.92 | 94.21 | 28.11 | 17.09 | 1.7 | 0.39 | 0.67 |
| **64** | 294.64 | 110.78 | 30.83 | 18.25 | 1.74 | 0.31 | 0.63 |
| **65** | 332.17 | 110.79 | 32.62 | 17.71 | 1.86 | 0.35 | 0.64 |
| **66** | 399.3 | 119.15 | 35.18 | 19.62 | 1.8 | 0.37 | 0.65 |
| **67** | 430.49 | 137.43 | 37.2 | 22.33 | 1.75 | 0.29 | 0.63 |
| **68** | 304.84 | 105.48 | 30.82 | 17.86 | 1.8 | 0.36 | 0.64 |
| **69** | 409.22 | 105.06 | 32.5 | 19.82 | 1.69 | 0.47 | 0.7 |
| **70** | 327.56 | 94.57 | 32.24 | 15.44 | 2.15 | 0.47 | 0.64 |
| **71** | 330.37 | 107.49 | 32.24 | 17.42 | 1.91 | 0.37 | 0.64 |
| **72** | - | - | - | - | - | - | - |
| **73** | 282.03 | 101.9 | 29.6 | 16.49 | 1.9 | 0.35 | 0.64 |
| **74** | - | - | - | - | - | - | - |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **75** | - | - | - | - | - | - | - |
| **76** | 353.42 | 101.69 | 31.03 | 18.26 | 1.75 | 0.44 | 0.69 |
| **77** | 408.5 | 112.76 | 35.96 | 18.68 | 2 | 0.41 | 0.64 |
| **78** | 328.53 | 110.87 | 31.65 | 18.87 | 1.73 | 0.35 | 0.65 |
| **79** | 361.98 | 106.76 | 32.15 | 19.13 | 1.72 | 0.41 | 0.67 |
| **80** | 305.16 | 111.61 | 32.17 | 17.35 | 1.9 | 0.32 | 0.62 |
| **81** | - | - | - | - | - | - | - |
| **82** | - | - | - | - | - | - | - |
| **83** | 443.76 | 120.49 | 34.59 | 21.33 | 1.66 | 0.39 | 0.69 |
| **84** | 384.02 | 109.54 | 34.85 | 17.28 | 2.09 | 0.41 | 0.64 |
| **85** | 532.06 | 139.93 | 39.99 | 23.93 | 1.71 | 0.35 | 0.65 |
| **86** | 358.78 | 117.96 | 33.78 | 18.97 | 1.81 | 0.33 | 0.63 |
| **87** | 353.87 | 127.17 | 33.92 | 20.44 | 1.71 | 0.29 | 0.63 |
| **88** | - | - | - | - | - | - | - |
| **89** | 302.55 | 110.41 | 33.04 | 16.07 | 2.13 | 0.32 | 0.6 |
| **90** | - | - | - | - | - | - | - |
| **91** | 359.98 | 113.89 | 32.88 | 19.13 | 1.8 | 0.36 | 0.65 |
| **92** | 377.08 | 118.28 | 35.46 | 18.38 | 2 | 0.35 | 0.61 |
| **93** | - | - | - | - | - | - | - |
| **94** | - | - | - | - | - | - | - |
| **95** | 320.09 | 112.69 | 30.54 | 20.15 | 1.58 | 0.33 | 0.66 |
| **96** | 351.56 | 112.22 | 34 | 18.44 | 1.95 | 0.36 | 0.63 |
| **97** | 416.57 | 134.12 | 37.89 | 20.24 | 1.95 | 0.3 | 0.61 |
| **98** | 339.24 | 129.37 | 33.82 | 19.31 | 1.78 | 0.27 | 0.62 |
| **99** | 440.18 | 123.95 | 35.28 | 22.18 | 1.62 | 0.36 | 0.67 |
| **100** | 349.58 | 128.99 | 33.94 | 19.68 | 1.8 | 0.27 | 0.62 |
| **101** | - | - | - | - | - | - | - |
| **102** | 369.42 | 107.89 | 32.32 | 19.28 | 1.75 | 0.41 | 0.68 |
| **103** | 465.36 | 122.01 | 35.53 | 22.04 | 1.67 | 0.4 | 0.69 |
| **104** | 374 | 114.3 | 33.25 | 20.12 | 1.68 | 0.37 | 0.66 |
| **105** | 291.4 | 83.61 | 27.92 | 16.06 | 1.78 | 0.52 | 0.69 |
| **106** | 494.14 | 146.55 | 40.49 | 21.44 | 1.92 | 0.31 | 0.62 |
| **107** | 336.16 | 135.37 | 34.69 | 19.66 | 1.84 | 0.24 | 0.6 |

**Appendix 1.7 Abaxial leaf epidemical cell scoring**

| | Area | Perimeter | Max. Length | Max. Breadth | Elongation | Roundness | Compactness |
|---|---|---|---|---|---|---|---|
| **1** | - | - | - | - | - | - | - |
| **2** | - | - | - | - | - | - | - |
| **3** | - | - | - | - | - | - | - |
| **4** | - | - | - | - | - | - | - |
| **5** | - | - | - | - | - | - | - |
| **6** | - | - | - | - | - | - | - |
| **7** | - | - | - | - | - | - | - |
| **8** | 2344 | 328 | 89 | 50 | 2 | 0 | 1 |
| **9** | 2206 | 432 | 116 | 53 | 27 | 0 | 1 |
| **10** | 2122 | 367 | 93 | 49 | 2 | 0 | 1 |
| **11** | 2775 | 456 | 108 | 60 | 2 | 0 | 1 |
| **12** | - | - | - | - | - | - | - |
| **13** | - | - | - | - | - | - | - |
| **14** | 2555 | 393 | 110 | 52 | 2 | 0 | 1 |
| **15** | 2930 | 482 | 133 | 57 | 25 | 0 | 1 |
| **16** | 3130 | 476 | 111 | 64 | 2 | 0 | 1 |
| **17** | - | - | - | - | - | - | - |
| **18** | 2217 | 334 | 93 | 47 | 2 | 0 | 1 |
| **19** | 2107 | 390 | 105 | 48 | 14 | 0 | 1 |
| **20** | - | - | - | - | - | - | - |
| **21** | 2059 | 304 | 88 | 45 | 2 | 0 | 1 |
| **22** | 2750 | 395 | 108 | 52 | 2 | 0 | 1 |
| **23** | 2419 | 451 | 101 | 62 | 2 | 0 | 1 |
| **24** | 2054 | 347 | 91 | 49 | 2 | 0 | 1 |
| **25** | 2705 | 395 | 99 | 59 | 2 | 0 | 1 |
| **26** | - | - | - | - | - | - | - |
| **27** | 2253 | 375 | 98 | 50 | 2 | 0 | 1 |
| **28** | 2783 | 424 | 110 | 55 | 2 | 0 | 1 |
| **29** | 2935 | 463 | 114 | 60 | 2 | 0 | 1 |
| **30** | 2396 | 391 | 106 | 54 | 14 | 0 | 1 |
| **31** | - | - | - | - | - | - | - |
| **32** | 2295 | 383 | 99 | 53 | 2 | 0 | 1 |
| **33** | 2039 | 362 | 99 | 50 | 8 | 0 | 1 |
| **34** | 2541 | 386 | 99 | 56 | 2 | 0 | 1 |
| **35** | 2266 | 412 | 116 | 50 | 22 | 0 | 1 |
| **36** | 2194 | 366 | 107 | 47 | 10 | 0 | 1 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **37** | 2363 | 358 | 96 | 52 | 2 | 0 | 1 |
| **38** | 2087 | 403 | 90 | 57 | 2 | 0 | 1 |
| **39** | 3594 | 476 | 119 | 67 | 2 | 0 | 1 |
| **40** | 2955 | 499 | 129 | 64 | 28 | 0 | 1 |
| **41** | 2077 | 403 | 98 | 49 | 2 | 0 | 1 |
| **42** | 2246 | 370 | 96 | 51 | 2 | 0 | 1 |
| **43** | 1299 | 177 | 49 | 28 | 5 | 1 | 1 |
| **44** | 1959 | 325 | 91 | 40 | 2 | 0 | 1 |
| **45** | 2358 | 338 | 94 | 51 | 2 | 0 | 1 |
| **46** | - | - | - | - | - | - | - |
| **47** | - | - | - | - | - | - | - |
| **48** | - | - | - | - | - | - | - |
| **49** | - | - | - | - | - | - | - |
| **50** | - | - | - | - | - | - | - |
| **51** | - | - | - | - | - | - | - |
| **52** | - | - | - | - | - | - | - |
| **53** | 2373 | 351 | 94 | 48 | 2 | 0 | 1 |
| **54** | 2085 | 317 | 84 | 48 | 2 | 0 | 1 |
| **55** | 3236 | 518 | 125 | 64 | 2 | 0 | 1 |
| **56** | - | - | - | - | - | - | - |
| **57** | - | - | - | - | - | - | - |
| **58** | - | - | - | - | - | - | - |
| **59** | - | - | - | - | - | - | - |
| **60** | 3122 | 432 | 113 | 57 | 2 | 0 | 1 |
| **61** | 2839 | 450 | 104 | 65 | 2 | 0 | 1 |
| **62** | 2054 | 328 | 89 | 45 | 2 | 0 | 1 |
| **63** | 2250 | 412 | 116 | 49 | 12 | 0 | 1 |
| **64** | 2057 | 362 | 91 | 53 | 2 | 0 | 1 |
| **65** | 2287 | 390 | 95 | 53 | 2 | 0 | 1 |
| **66** | 2569 | 359 | 103 | 47 | 2 | 0 | 1 |
| **67** | - | - | - | - | - | - | - |
| **68** | - | - | - | - | - | - | - |
| **69** | 1880 | 296 | 80 | 48 | 2 | 0 | 1 |
| **70** | 2755 | 442 | 120 | 58 | 23 | 0 | 1 |
| **71** | 2320 | 405 | 100 | 52 | 2 | 0 | 1 |
| **72** | 2065 | 259 | 77 | 46 | 2 | 0 | 1 |
| **73** | - | - | - | - | - | - | - |
| **74** | 2574 | 414 | 108 | 53 | 2 | 0 | 1 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **75** | - | - | - | - | - | - | - |
| **76** | 2008 | 395 | 111 | 47 | 28 | 0 | 1 |
| **77** | 2589 | 418 | 124 | 49 | 24 | 0 | 1 |
| **78** | 1601 | 279 | 78 | 41 | 2 | 0 | 1 |
| **79** | 1996 | 383 | 109 | 47 | 26 | 0 | 1 |
| **80** | - | - | - | - | - | - | - |
| **81** | - | - | - | - | - | - | - |
| **82** | 3048 | 441 | 112 | 60 | 2 | 0 | 1 |
| **83** | - | - | - | - | - | - | - |
| **84** | - | - | - | - | - | - | - |
| **85** | - | - | - | - | - | - | - |
| **86** | - | - | - | - | - | - | - |
| **87** | - | - | - | - | - | - | - |
| **88** | - | - | - | - | - | - | - |
| **89** | - | - | - | - | - | - | - |
| **90** | - | - | - | - | - | - | - |
| **91** | - | - | - | - | - | - | - |
| **92** | - | - | - | - | - | - | - |
| **93** | - | - | - | - | - | - | - |
| **94** | - | - | - | - | - | - | - |
| **95** | - | - | - | - | - | - | - |
| **96** | - | - | - | - | - | - | - |
| **97** | - | - | - | - | - | - | - |
| **98** | - | - | - | - | - | - | - |
| **99** | - | - | - | - | - | - | - |
| **100** | 1757 | 243 | 68 | 41 | 2 | 1 | 1 |
| **101** | - | - | - | - | - | - | - |
| **102** | - | - | - | - | - | - | - |
| **103** | - | - | - | - | - | - | - |
| **104** | - | - | - | - | - | - | - |
| **105** | - | - | - | - | - | - | - |
| **106** | - | - | - | - | - | - | - |
| **107** | - | - | - | - | - | - | - |

**Appendix 1.8 Abaxial petal epidemical cell scoring**

| | Area | Perimeter | Max. Length | Max. Breadth | Elongation | Roundness | Compactness |
|---|---|---|---|---|---|---|---|
| **1** | 354.43 | 82.55 | 26.61 | 18.55 | 1.44 | 0.66 | 0.8 |
| **2** | 467.5 | 98.76 | 35.13 | 20.46 | 1.41 | 0.71 | 0.79 |
| **3** | 708.52 | 125.84 | 44.69 | 26.11 | 6.67 | 0.73 | 0.82 |
| **4** | 434 | 105.34 | 38.24 | 20.53 | 1.3 | 0.72 | 0.82 |
| **5** | - | - | - | - | - | - | - |
| **6** | - | - | - | - | - | - | - |
| **7** | - | - | - | - | - | - | - |
| **8** | 526.95 | 97.29 | 31.64 | 23.15 | 1.38 | 0.72 | 0.82 |
| **9** | - | - | - | - | - | - | - |
| **10** | 753.05 | 131.76 | 46.06 | 26.55 | 4.94 | 0.7 | 0.8 |
| **11** | 631.97 | 118.79 | 42.08 | 24.33 | 1.42 | 0.68 | 0.78 |
| **12** | - | - | - | - | - | - | - |
| **13** | - | - | - | - | - | - | - |
| **14** | - | - | - | - | - | - | - |
| **15** | 504.86 | 105.81 | 38.24 | 21.16 | 4.28 | 0.71 | 0.79 |
| **16** | -10.87 | -294.15 | -339.23 | -348.76 | -361.59 | -362.83 | -362.76 |
| **17** | 715.5 | 110.81 | 37.28 | 26.41 | 1.45 | 0.74 | 0.82 |
| **18** | - | - | - | - | - | - | - |
| **19** | 522.09 | 114.36 | 42.29 | 21.01 | 12.6 | 0.66 | 0.75 |
| **20** | 387.82 | 94.98 | 35.13 | 16.93 | 6.03 | 0.66 | 0.74 |
| **21** | 560.83 | 114.45 | 41.27 | 23.09 | 1.35 | 0.69 | 0.78 |
| **22** | - | - | - | - | - | - | - |
| **23** | 412.58 | 83.2 | 27.32 | 20.19 | 1.38 | 0.74 | 0.83 |
| **24** | 712.03 | 110.01 | 35.94 | 26.66 | 1.37 | 0.73 | 0.83 |
| **25** | 414.81 | 100.22 | 35.64 | 19.57 | 9.12 | 0.67 | 0.78 |
| **26** | - | - | - | - | - | - | - |
| **27** | 795.56 | 137.92 | 49.96 | 27.6 | 13.84 | 0.7 | 0.79 |
| **28** | 476.67 | 103.9 | 37.53 | 21.34 | 9.97 | 0.72 | 0.81 |
| **29** | - | - | - | - | - | - | - |
| **30** | 443.76 | 85.59 | 28 | 21.47 | 1.32 | 0.75 | 0.84 |
| **31** | 630.63 | 126.25 | 47.14 | 23.99 | 14.9 | 0.7 | 0.77 |
| **32** | 413.22 | 101 | 36.56 | 19.44 | 11.86 | 0.72 | 0.81 |
| **33** | 432.05 | 105.14 | 38.1 | 19.37 | 7.47 | 0.71 | 0.82 |
| **34** | 664.45 | 130.16 | 46.82 | 24.5 | 14.11 | 0.68 | 0.78 |
| **35** | - | - | - | - | - | - | - |
| **36** | 440.65 | 100.9 | 35.44 | 20.06 | 4.18 | 0.68 | 0.8 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **37** | 444.54 | 88.5 | 29.37 | 20.59 | 1.46 | 0.7 | 0.8 |
| **38** | 460.36 | 112.67 | 42.31 | 19.6 | 15.44 | 0.68 | 0.78 |
| **39** | 689.38 | 110.73 | 35.68 | 26.88 | 1.35 | 0.7 | 0.83 |
| **40** | 515.79 | 116.51 | 42 | 21.31 | 7.3 | 0.68 | 0.79 |
| **41** | - | - | - | - | - | - | - |
| **42** | 780.83 | 136.59 | 49.37 | 26.98 | 1.36 | 0.7 | 0.79 |
| **43** | 456.21 | 89.01 | 29.41 | 21.26 | 1.41 | 0.71 | 0.81 |
| **44** | 856.33 | 124.95 | 40.22 | 29.82 | 1.36 | 0.7 | 0.82 |
| **45** | 958.68 | 132.95 | 46.26 | 28.82 | 1.65 | 0.68 | 0.76 |
| **46** | 687.3 | 126.43 | 43.83 | 26.16 | 1.34 | 0.67 | 0.79 |
| **47** | 683.53 | 107.83 | 34.89 | 27.17 | 1.3 | 0.73 | 0.84 |
| **48** | 693.11 | 132.13 | 47.86 | 25.34 | 14.38 | 0.69 | 0.79 |
| **49** | 426.86 | 99.31 | 35.75 | 19.03 | 4.34 | 0.69 | 0.78 |
| **50** | 369.77 | 95.41 | 34.38 | 18.58 | 10.74 | 0.69 | 0.8 |
| **51** | 523.7 | 117.1 | 42.79 | 22.31 | 13.87 | 0.67 | 0.78 |
| **52** | 349.82 | 93.02 | 33.35 | 17.52 | 5.99 | 0.66 | 0.79 |
| **53** | 549.73 | 93.89 | 31.73 | 23.22 | 1.39 | 0.77 | 0.83 |
| **54** | 797.71 | 140.52 | 50.19 | 27.38 | 13.71 | 0.67 | 0.78 |
| **55** | 589.95 | 127.76 | 46.59 | 23.6 | 16.07 | 0.66 | 0.79 |
| **56** | - | - | - | - | - | - | - |
| **57** | 543.88 | 118.32 | 42.5 | 23.04 | 14.13 | 0.69 | 0.81 |
| **58** | 503.57 | 114.57 | 42.27 | 21.08 | 14.56 | 0.7 | 0.8 |
| **59** | 439.48 | 85.96 | 28.7 | 19.73 | 1.5 | 0.75 | 0.82 |
| **60** | - | - | - | - | - | - | - |
| **61** | 677 | 131.62 | 48.43 | 24.38 | 14.25 | 0.65 | 0.75 |
| **62** | 887 | 121.12 | 38.65 | 31.64 | 1.25 | 0.75 | 0.86 |
| **63** | 618.91 | 125.34 | 45.9 | 23.76 | 14.98 | 0.71 | 0.8 |
| **64** | 495.8 | 92.3 | 30.79 | 22.19 | 1.41 | 0.72 | 0.81 |
| **65** | 630.49 | 107.39 | 33.94 | 25.99 | 1.32 | 0.69 | 0.83 |
| **66** | - | - | - | - | - | - | - |
| **67** | 600.86 | 99.74 | 32.85 | 25.07 | 1.32 | 0.75 | 0.84 |
| **68** | 581.48 | 119.72 | 43.87 | 23.25 | 14.59 | 0.73 | 0.82 |
| **69** | 473.27 | 88.86 | 29.23 | 22.66 | 1.33 | 0.74 | 0.84 |
| **70** | 602.39 | 118.94 | 43.67 | 23.78 | 13.33 | 0.73 | 0.8 |
| **71** | 471.12 | 116.09 | 43.49 | 20.64 | 1.44 | 0.68 | 0.78 |
| **72** | 603.24 | 102.33 | 33.57 | 24.72 | 1.39 | 0.72 | 0.82 |
| **73** | 386.32 | 81.3 | 26.12 | 19.89 | 1.32 | 0.72 | 0.84 |
| **74** | 593.47 | 100.26 | 32.92 | 24.81 | 1.36 | 0.73 | 0.83 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **75** | - | - | - | - | - | - | - |
| **76** | 686.36 | 106.45 | 34.86 | 26.56 | 1.32 | 0.76 | 0.85 |
| **77** | 531.77 | 111.48 | 40.75 | 20.89 | 6.09 | 0.68 | 0.77 |
| **78** | 423.98 | 100.26 | 36.25 | 19.6 | 1.46 | 0.69 | 0.79 |
| **79** | 591.27 | 100.65 | 33.14 | 24.27 | 1.4 | 0.72 | 0.82 |
| **80** | 330.14 | 76.87 | 24.46 | 18.36 | 1.35 | 0.7 | 0.84 |
| **81** | - | - | - | - | - | - | - |
| **82** | 917.09 | 125.58 | 42.12 | 29.34 | 1.44 | 0.73 | 0.81 |
| **83** | 538.21 | 98.56 | 32.05 | 24.26 | 1.34 | 0.7 | 0.82 |
| **84** | 538.54 | 95.2 | 31.19 | 23.14 | 1.35 | 0.74 | 0.83 |
| **85** | 429.39 | 85.52 | 27.79 | 20.88 | 1.35 | 0.74 | 0.84 |
| **86** | 2193.93 | 193.66 | 63.08 | 48.17 | 1.33 | 0.73 | 0.83 |
| **87** | 332.05 | 72.95 | 23.34 | 18.35 | 1.29 | 0.78 | 0.88 |
| **88** | 886.8 | 123.27 | 39.84 | 30.93 | 1.29 | 0.72 | 0.84 |
| **89** | 316.57 | 73.02 | 24.3 | 16.76 | 1.47 | 0.74 | 0.82 |
| **90** | - | - | - | - | - | - | - |
| **91** | 694.95 | 107.8 | 34.39 | 27.62 | 1.26 | 0.74 | 0.86 |
| **92** | - | - | - | - | - | - | - |
| **93** | - | - | - | - | - | - | - |
| **94** | 840.6 | 119.48 | 38.91 | 30.04 | 1.3 | 0.74 | 0.84 |
| **95** | - | - | - | - | - | - | - |
| **96** | 793.56 | 115.11 | 38.01 | 28.37 | 1.35 | 0.73 | 0.83 |
| **97** | 495.44 | 91.18 | 29.95 | 22.68 | 1.34 | 0.73 | 0.83 |
| **98** | 513.43 | 93.8 | 31.48 | 21.82 | 1.45 | 0.73 | 0.81 |
| **99** | 770.92 | 121.78 | 38.51 | 27.47 | 1.42 | 0.67 | 0.81 |
| **100** | 499.62 | 91.7 | 30.34 | 22.28 | 1.42 | 0.73 | 0.82 |
| **101** | - | - | - | - | - | - | - |
| **102** | 647.91 | 125.05 | 44.39 | 24.26 | 12.8 | 0.7 | 0.8 |
| **103** | 439.29 | 96.96 | 34.68 | 20.27 | 9.33 | 0.74 | 0.82 |
| **104** | 770.39 | 112.32 | 36.48 | 27.86 | 1.32 | 0.75 | 0.85 |
| **105** | 882.35 | 124.72 | 43.19 | 27.28 | 1.64 | 0.69 | 0.76 |
| **106** | 630.08 | 101.97 | 34.86 | 25.15 | 1.43 | 0.76 | 0.81 |
| **107** | 403.38 | 99.49 | 36.19 | 19.29 | 6.38 | 0.7 | 0.79 |

**Appendix 1.9 Trichome densities**

| | Abax_lf | Abax_long | Abax_pet | Adax_lf | Adax_long |
|---|---|---|---|---|---|
| **1** | 30 | 0 | - | 11 | 2 |
| **2** | 0 | - | 212 | 8 | 3 |
| **3** | 0 | - | 104 | - | - |
| **4** | - | - | 164 | - | - |
| **5** | 37 | 3 | 31 | 37 | 3 |
| **6** | 100 | - | - | - | - |
| **7** | 0 | - | 78 | 28 | 2 |
| **8** | 0 | - | - | - | - |
| **9** | 6 | 0 | 60 | 59 | 3 |
| **10** | 0 | - | 108 | 41 | 0 |
| **11** | 26 | 0 | 104 | 27 | 1 |
| **12** | - | - | 200 | - | - |
| **13** | - | - | - | - | - |
| **14** | 6 | 1 | 120 | - | - |
| **15** | 0 | - | 55 | 25 | 3 |
| **16** | 0 | - | 3 | 12 | 3 |
| **17** | 0 | - | 51 | 3 | 3 |
| **18** | 0 | - | 55 | 2 | 3 |
| **19** | 0 | - | 19 | 41 | 1 |
| **20** | 0 | - | - | 28 | 3 |
| **21** | 0 | - | 25 | 0 | - |
| **22** | 0 | - | - | 0 | - |
| **23** | 0 | - | 19 | 39 | 0 |
| **24** | 0 | - | 60 | 33 | 2 |
| **25** | 0 | - | 64 | 2 | 3 |
| **26** | 0 | - | 80 | 0 | - |
| **27** | 0 | - | 0 | 25 | 2 |
| **28** | 0 | - | 140 | 3 | 3 |
| **29** | 0 | - | 140 | 0 | - |
| **30** | 0 | - | 23 | 8 | 0 |
| **31** | 13 | 0 | 140 | 43 | 1 |
| **32** | 0 | - | 4 | 3 | 3 |
| **33** | 0 | - | 0 | 0 | - |
| **34** | 0 | - | 52 | 22 | 3 |
| **35** | 6 | 3 | 35 | 0 | - |
| **36** | 0 | - | 200 | 19 | 3 |

| | | | | | |
|---|---|---|---|---|---|
| **37** | 0 | - | 59 | 0 | - |
| **38** | 0 | - | 50 | 61 | 0 |
| **39** | 0 | - | 70 | 2 | 3 |
| **40** | 24 | 1 | 200 | 47 | 2 |
| **41** | 0 | - | - | 48 | 2 |
| **42** | 0 | - | - | 11 | 3 |
| **43** | 0 | - | 200 | 24 | 1 |
| **44** | 0 | - | 0 | 40 | 3 |
| **45** | 0 | - | 130 | 31 | 2 |
| **46** | 0 | - | 90 | 27 | 0 |
| **47** | 0 | - | 0 | 0 | - |
| **48** | 0 | - | 17 | 0 | - |
| **49** | 6 | 3 | 470 | 47 | 2 |
| **50** | 0 | - | 14 | 41 | 2 |
| **51** | 0 | - | 106 | 19 | 2 |
| **52** | 0 | - | 38 | 0 | - |
| **53** | 0 | - | 40 | 20 | 1 |
| **54** | 0 | - | 104 | 18 | 0 |
| **55** | 2 | 3 | 208 | 80 | 2 |
| **56** | - | - | - | - | - |
| **57** | 0 | - | 10 | 44 | 0 |
| **58** | 0 | - | - | 43 | 3 |
| **59** | 0 | - | 88 | 2 | 0 |
| **60** | - | - | 188 | 86 | 2 |
| **61** | 0 | - | 176 | 27 | 0 |
| **62** | 0 | - | 11 | 16 | 2 |
| **63** | 0 | - | 32 | 32 | 2 |
| **64** | 13 | 0 | 84 | 44 | 1 |
| **65** | 0 | - | - | 18 | 3 |
| **66** | 2 | 0 | 172 | 36 | 2 |
| **67** | 3 | 0 | - | 30 | 1 |
| **68** | 0 | - | 120 | 36 | 1 |
| **69** | 0 | - | 23 | 8 | 3 |
| **70** | 0 | - | 200 | 0 | - |
| **71** | 0 | - | 32 | 37 | 2 |
| **72** | 0 | - | 0 | - | - |
| **73** | 0 | - | 2 | 0 | - |
| **74** | 0 | - | 30 | 0 | - |

| | | | | | |
|---|---|---|---|---|---|
| **75** | - | - | - | - | - |
| **76** | 0 | - | 34 | 17 | 2 |
| **77** | 0 | - | - | 7 | 3 |
| **78** | 3 | 0 | 44 | 24 | 2 |
| **79** | 0 | - | 140 | 51 | 2 |
| **80** | 3 | 0 | 50 | 20 | 2 |
| **81** | - | - | - | - | - |
| **82** | 0 | - | 140 | - | - |
| **83** | 1 | 0 | 180 | 68 | 2 |
| **84** | - | - | 50 | - | - |
| **85** | 0 | - | 112 | 8 | 2 |
| **86** | 0 | - | 78 | 38 | 2 |
| **87** | 5 | 0 | 100 | 44 | 2 |
| **88** | - | - | 128 | 19 | 2 |
| **89** | 0 | - | 31 | 0 | - |
| **90** | - | - | - | - | - |
| **91** | 0 | - | 47 | 32 | 2 |
| **92** | 0 | - | 41 | 6 | 2 |
| **93** | 0 | - | 10 | - | - |
| **94** | - | - | 18 | - | - |
| **95** | 0 | - | 116 | 6 | 2 |
| **96** | 0 | - | 140 | 0 | - |
| **97** | 23 | 2 | 136 | 50 | 3 |
| **98** | 0 | - | 42 | 30 | 0 |
| **99** | 0 | - | 120 | 31 | 2 |
| **100** | 0 | - | - | 22 | 2 |
| **101** | - | - | - | - | - |
| **102** | - | - | - | - | - |
| **103** | - | - | - | - | - |
| **104** | - | - | - | - | - |
| **105** | - | - | - | - | - |
| **106** | - | - | - | - | - |
| **107** | - | - | - | - | - |

**Appendix 2 Principal Components data from m/m and c/m set.**

| | Mol_PC1 | Mol_PC2 | Mol_PC3 | Mol_PC4 | Ch_mol_PC1 | Ch_mol_PC2 | Ch_mol_PC3 | Ch_mol_PC4 |
|---|---|---|---|---|---|---|---|---|
| **1** | -4.36 | -0.37 | -1.75 | 0.06 | 1.38 | -2.68 | -0.41 | 0.97 |
| **2** | -9.68 | 5.38 | -1.16 | -0.77 | 13.41 | -3.37 | 3.42 | -1.02 |
| **3** | 4.54 | 0.4 | -0.49 | 0.03 | 3.02 | -1.75 | 0.24 | 0.28 |
| **4** | -0.53 | -0.23 | 0.52 | 0.07 | 1.75 | -0.95 | -1.71 | 0.67 |
| **5** | 3.91 | -0.22 | 0.19 | 0.27 | 1.74 | -1.08 | -0.55 | 1.5 |
| **6** | -4.87 | -1.02 | -1.25 | 0.23 | -0.82 | -2.18 | -0.17 | 0.84 |
| **7** | 2.64 | -0.65 | -0.96 | -0.98 | 0.8 | -1.9 | 0.46 | -1.42 |
| **8** | -3.38 | -0.67 | 0.28 | -0.47 | 0.82 | -0.97 | -0.86 | 0.15 |
| **9** | 7.39 | -0.66 | -0.29 | -0.65 | 0.82 | -1.59 | -1.65 | -0.37 |
| **10** | -3.45 | -0.04 | -0.24 | -0.72 | 2.11 | -1.41 | 0.25 | -0.56 |
| **11** | -10.58 | -0.36 | -0.14 | 0.11 | 1.46 | -1.57 | -2.18 | 1.28 |
| **12** | 3.12 | -0.5 | 0.87 | 0.41 | 1.19 | -0.47 | -1.2 | 1.86 |
| **13** | 0.8 | 3.56 | -0.7 | 1.63 | 9.62 | -2.62 | 1.88 | 3.42 |
| **14** | -4.77 | -0.93 | -0.61 | 0.18 | 0.25 | -1.54 | -0.22 | 1.3 |
| **15** | -6.19 | 0.59 | 1.26 | -1.07 | 3.47 | -0.34 | 0.34 | -1.22 |
| **16** | -4.07 | 0.15 | 0.35 | -0.02 | 2.53 | -1.01 | -0.44 | 0.71 |
| **17** | -0.04 | 0.52 | 1.29 | 0.61 | 3.32 | -0.47 | -0.73 | 1.66 |
| **18** | 4.3 | -0.22 | 2.53 | -0.45 | 1.85 | 0.78 | -1.94 | -0.16 |
| **19** | -5.73 | -0.69 | -1.32 | -0.24 | 0.73 | -2.27 | -0.5 | 0.41 |
| **20** | 3.73 | 0.42 | 1.42 | -0.6 | 3.13 | -0.26 | -1.23 | -0.76 |
| **21** | 1.3 | -0.83 | 0.56 | -0.19 | 0.49 | -0.61 | -0.81 | 0.25 |
| **22** | - | - | - | - | - | - | - | - |
| **23** | 4.89 | -0.72 | -0.78 | -0.18 | 0.68 | -1.81 | -0.77 | 0.55 |
| **24** | -0.9 | 0.23 | 0.39 | 0.26 | 2.71 | -1.21 | -1.54 | 1.49 |
| **25** | 1.35 | -0.66 | -0.85 | -0.04 | 0.78 | -1.86 | -0.14 | 0.44 |
| **26** | -4.13 | -1.15 | 0.29 | -0.23 | -0.19 | -0.69 | -0.48 | 0.15 |
| **27** | -3.78 | 0.98 | 0.96 | -0.78 | 4.27 | -0.61 | 0.78 | -0.46 |
| **28** | -10.09 | 0.07 | 1.62 | -0.83 | 2.2 | -0.33 | -0.71 | 3.31 |
| **29** | 6 | -0.57 | 1 | 0.1 | 1.06 | -0.58 | -2 | 0.92 |
| **30** | 12.43 | -0.86 | 0.51 | 0.61 | 0.42 | -0.72 | -1.07 | 1.53 |
| **31** | 11.3 | 0.62 | -1.72 | -0.83 | 3.46 | -3.06 | -1.1 | -1.18 |
| **32** | 9.93 | -0.23 | 0.17 | 0.26 | 1.73 | -1.37 | -2.04 | 1.13 |
| **33** | 2.76 | -0.53 | -1.76 | 0.14 | 1.04 | -2.65 | -0.04 | 0.83 |
| **34** | 4.47 | -0.2 | -0.55 | -0.73 | 1.76 | -1.62 | 0.46 | -0.92 |
| **35** | 0.82 | -0.28 | 0.09 | -0.56 | 1.62 | -1 | 0.63 | -0.88 |
| **36** | 0.27 | -0.23 | -1.27 | -0.43 | 1.7 | -2.36 | -0.45 | -0.26 |

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|---|---|---|---|---|---|---|---|---|
| **37** | -12.97 | 0.23 | -0.12 | -1.1 | 2.66 | -1.41 | 0.94 | -1.32 |
| **38** | 0.57 | 0.01 | -0.6 | -1.06 | 2.19 | -1.83 | 0.04 | -0.9 |
| **39** | -0.16 | 0.27 | 1.04 | 1.87 | 2.79 | -0.35 | 0.43 | 3.87 |
| **40** | -0.98 | 0.2 | 0.38 | -0.66 | 2.62 | -1.25 | -1.28 | -0.09 |
| **41** | 5.36 | -0.47 | 0.83 | -0.69 | 1.26 | -0.53 | -0.95 | -1.22 |
| **42** | 15.96 | -0.38 | -0.73 | 1.79 | 1.39 | -1.71 | 0.18 | 4.43 |
| **43** | 4.6 | 0.35 | 0.32 | -0.54 | 2.94 | -1.23 | -1.27 | -0.17 |
| **44** | - | - | - | - | - | - | - | - |
| **45** | 5.74 | 0.08 | -0.69 | -0.54 | 2.34 | -2 | -0.31 | -0.54 |
| **46** | -0.03 | 0.39 | -1.78 | -0.12 | 2.98 | -2.99 | -0.43 | 0.21 |
| **47** | 5.29 | 0.72 | 0.32 | 0.04 | 3.71 | -1.3 | -0.33 | 0.84 |
| **48** | 2.73 | -0.52 | -0.81 | -0.02 | 1.09 | -1.63 | 1.13 | 0.62 |
| **49** | 3.82 | -0.08 | -0.91 | -0.13 | 2.01 | -2.2 | -1.01 | 0.39 |
| **50** | 2.94 | -0.26 | -0.08 | -0.15 | 1.65 | -1.3 | -0.18 | 0.53 |
| **51** | -6.04 | -0.55 | 0.21 | -0.95 | 1.06 | -0.85 | -0.1 | -0.93 |
| **52** | -12.87 | 0.19 | 1.09 | -0.03 | 2.63 | -0.65 | -0.98 | 0.54 |
| **53** | -4.64 | -0.31 | 0.42 | -0.33 | 1.57 | -1.14 | -2.07 | 0.43 |
| **54** | -0.63 | 1.43 | 0.53 | 0.5 | 5.23 | -1.37 | -1.25 | 2.19 |
| **55** | 1.93 | -0.05 | 1.3 | -0.94 | 2.13 | -0.15 | -0.48 | -0.67 |
| **56** | - | - | - | - | - | - | - | - |
| **57** | 2.65 | -0.26 | -0.21 | -0.1 | 1.66 | -1.31 | 0.22 | 0.32 |
| **58** | -5.19 | -0.79 | -0.18 | -0.33 | 0.54 | -1.16 | 0 | 0.7 |
| **59** | -4.53 | -0.51 | -0.91 | -0.72 | 1.11 | -1.93 | -0.32 | -0.7 |
| **60** | 6.98 | -0.66 | 0.86 | -0.05 | 0.85 | -0.76 | -2.74 | 0.77 |
| **61** | 13.18 | 4.43 | -0.96 | -0.04 | 11.44 | -2.88 | 2.27 | 0.72 |
| **62** | -11.75 | -0.54 | -0.57 | 0.15 | 1.07 | -1.8 | -1.38 | 1.58 |
| **63** | -7.4 | 0.05 | -0.51 | -0.14 | 2.29 | -1.84 | -1.19 | 0.99 |
| **64** | -8.41 | -0.04 | -1.34 | 0.29 | 2.08 | -2.48 | -0.36 | 1.89 |
| **65** | -9.59 | -0.3 | 0.11 | 1.07 | 1.59 | -1.2 | -1.37 | 3.25 |
| **66** | 3.1 | 0.17 | -0.65 | 0.38 | 2.53 | -2.03 | -1 | 2.16 |
| **67** | -7.26 | -1.31 | -0.18 | -0.11 | -0.54 | -1.03 | -0.28 | 1.15 |
| **68** | 4.02 | 0.45 | 0.33 | 0.33 | 3.14 | -1.01 | 0.61 | 1.55 |
| **69** | 4.06 | -0.15 | 2.28 | 0.36 | 1.96 | 0.39 | -2.19 | 1.18 |
| **70** | 0.56 | -1.28 | 1.21 | -0.16 | -0.45 | 0.25 | 0.25 | 0.19 |
| **71** | 1.07 | -1 | 0.25 | -0.4 | 0.12 | -0.92 | -0.89 | 0.32 |
| **72** | -1.22 | 0.82 | -0.41 | -0.17 | 3.9 | -1.56 | 1.74 | 0.23 |
| **73** | -1.52 | 1.5 | 2.26 | 0.17 | 5.42 | -0.2 | -3 | 2.04 |
| **74** | -1.38 | 0.4 | 0.6 | 0.36 | 3.07 | -1.07 | -1.27 | 1.61 |

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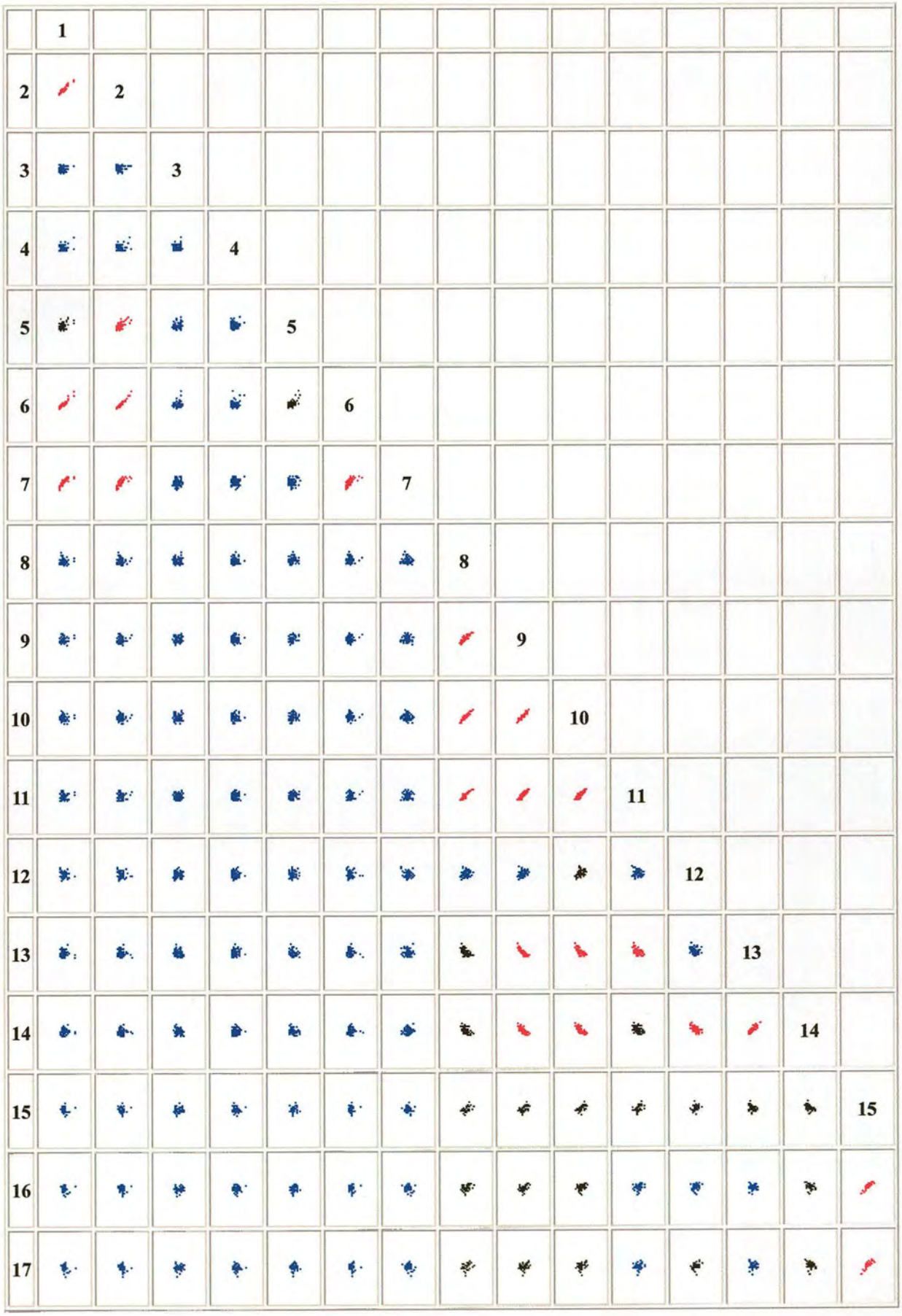
|---|---|---|---|---|---|---|---|---|
| **75** | - | - | - | - | - | - | - | - |
| **76** | -4.95 | 0.04 | 0.51 | 0.17 | 2.33 | -0.98 | -1.29 | 1.87 |
| **77** | 2.77 | 0.43 | 0.59 | -0.23 | 3.12 | -0.81 | 0.54 | 0.17 |
| **78** | -1.79 | 0.38 | -1.08 | -0.57 | 2.96 | -2.34 | -0.06 | -0.58 |
| **79** | 5.3 | -0.2 | -0.04 | 0.2 | 1.8 | -1.32 | -0.6 | 0.48 |
| **80** | 3.81 | -0.13 | -1.36 | -0.27 | 1.9 | -2.56 | -1.23 | 0.87 |
| **81** | - | - | - | - | - | - | - | - |
| **82** | -8.69 | -0.89 | -0.5 | -0.12 | 0.32 | -1.34 | 0.38 | 0.78 |
| **83** | 4.93 | 0.5 | 1.13 | 0.42 | 3.3 | -0.52 | -0.71 | 1.47 |
| **84** | -0.59 | 1.05 | 0.3 | 1.54 | 4.42 | -1.59 | -1.65 | 4.41 |
| **85** | -0.15 | -0.78 | 0.74 | 0.32 | 0.6 | -0.62 | -1.53 | 1.63 |
| **86** | -1.9 | 0.25 | 1.27 | -0.6 | 2.77 | -0.39 | -1.31 | -0.03 |
| **87** | 5.19 | 0.12 | -0.08 | 0.21 | 2.43 | -1.41 | -0.34 | 1.5 |
| **88** | -11.09 | 0.99 | 1.38 | 0.86 | 4.34 | -0.64 | -1.79 | 3.04 |
| **89** | 3.83 | -1.29 | 1.23 | -0.06 | -0.45 | 0.2 | -0.33 | 0.81 |
| **90** | 1.32 | -1.14 | -0.65 | 0 | -0.2 | -1.32 | 0.69 | 0.73 |
| **91** | 3.48 | -0.13 | -0.99 | 0.19 | 1.89 | -1.97 | 0.48 | 0.9 |
| **92** | -5.1 | -0.83 | -2.32 | -0.14 | 0.39 | -2.97 | 0.34 | 0.47 |
| **93** | 3.92 | 0.03 | -1.92 | 0.32 | 2.19 | -2.73 | 1.27 | 1.33 |
| **94** | 4.29 | -0.51 | -0.16 | 0.13 | 1.14 | -1.48 | -1.24 | 0.45 |
| **95** | 1.49 | 0.83 | -0.13 | -0.53 | 3.95 | -1.75 | -0.99 | -0.27 |
| **96** | 2.37 | -1.02 | 0.71 | 0.27 | 0.1 | -0.38 | -0.73 | 1.17 |
| **97** | -2.08 | 0.55 | -0.23 | 0.61 | 3.36 | -1.8 | -1.3 | 1.82 |
| **98** | -9.27 | -0.19 | -0.86 | 0.54 | 1.81 | -2.27 | -2.35 | 2.44 |
| **99** | 0.64 | -0.08 | -0.22 | -0.71 | 2.05 | -1.78 | -2.5 | -0.16 |
| **100** | 1.1 | 0.03 | 1.13 | -0.98 | 2.31 | -0.36 | -0.43 | -1.04 |
| **101** | -2.97 | -0.23 | 1.28 | 0.12 | 1.77 | -0.42 | -1.96 | 1.31 |
| **102** | 1.31 | 0.71 | 0.56 | -0.65 | 3.7 | -0.75 | 1.27 | -0.63 |
| **103** | -8.86 | -0.1 | 0.26 | 0.97 | 2.01 | -1.1 | -0.62 | 2.6 |
| **104** | 4 | -0.33 | -0.46 | 2.1 | 1.52 | -1.49 | -0.23 | 4.6 |
| **105** | 5.42 | -0.04 | -0.25 | -0.74 | 2.11 | -1.47 | -0.18 | -0.44 |
| **106** | -4.47 | 0.76 | -1.05 | -0.16 | 3.75 | -1.99 | 2.42 | 0.27 |
| **107** | 0.63 | -0.34 | 0.02 | 0.99 | 1.5 | -1.42 | -1.59 | 3.12 |

**Appendix 3 Correlation between two traits.**

1. Leaf Area
2. Leaf Perimeter
3. Leaf Centroid-x
4. Leaf Centroid-y
5. Leaf Circularity
6. Leaf Max. Length
7. Leaf Max. Breadth
8. lupperleaf_Area
9. lupperleaf_Perimeter
10. lupperleaf_Major_Axis_Length
11. lupperleaf_Minor_Axis_Length
12. lupperleaf_Elongation
13. lupperleaf_Roundness
14. lupperleaf_Compactness
15. BK_Area
16. BK_Perimeter
17. BK_MajorAxisLength
18. BK_MinorAxisLength
19. BK_Elongation
20. BK_Roundness
21. BK_Compactness
22. F-1
23. F-2
24. F-3
25. F-4
26. F-5
27. F-6
28. F-7
29. F-8
30. F-9
31. F-10
32. F-11
33. P-Area
34. P-Perimeter
35. P-Centroid-x
36. P-Centroid-y
37. P-Circularity
38. P-MajorAxis-Length
39. P-Breadth
40. Petal_Imprint_Area
41. Petal_Imprint_Perimeter
42. Petal_Imprint_Major_Axis_Length
43. Petal_Imprint_Minor_Axis_Length
44. Petal_Imprint_Elongation
45. Petal_Imprint_Roundness
46. Petal_Imprint_Compactness
47. flowering-node
48. day-to-flower
49. total-length
50. branch-no
51. node-diameter
52. Red
53. Yellow
54. pigment-analysis
55. Abax_lf
56. Abax_long
57. Abax_lf.BKArea
58. Abax_long.BKArea
59. Abax_pet
60. Adax_lf
61. Adax_long
62. Adax_lf.area
63. Adax_long.area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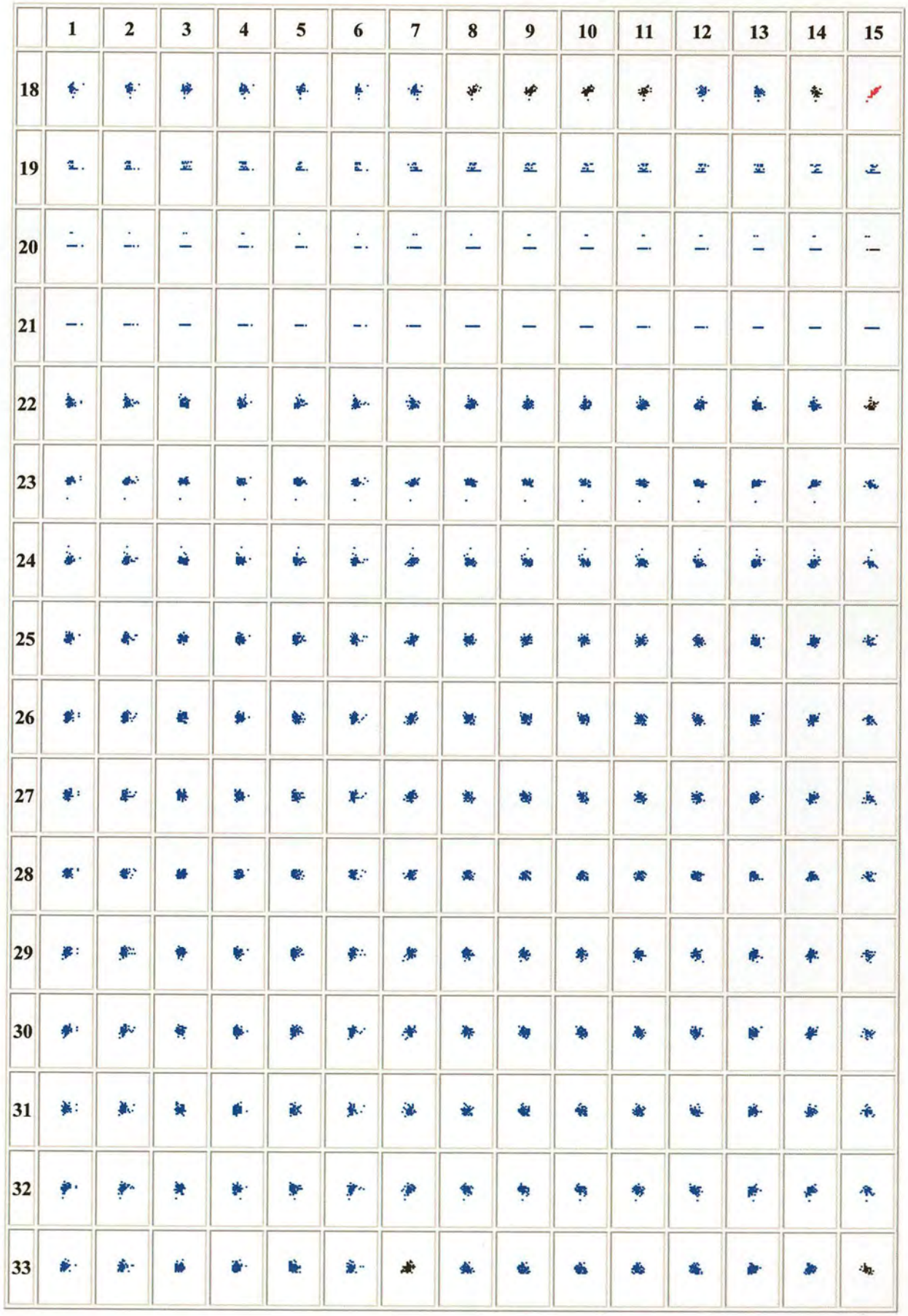
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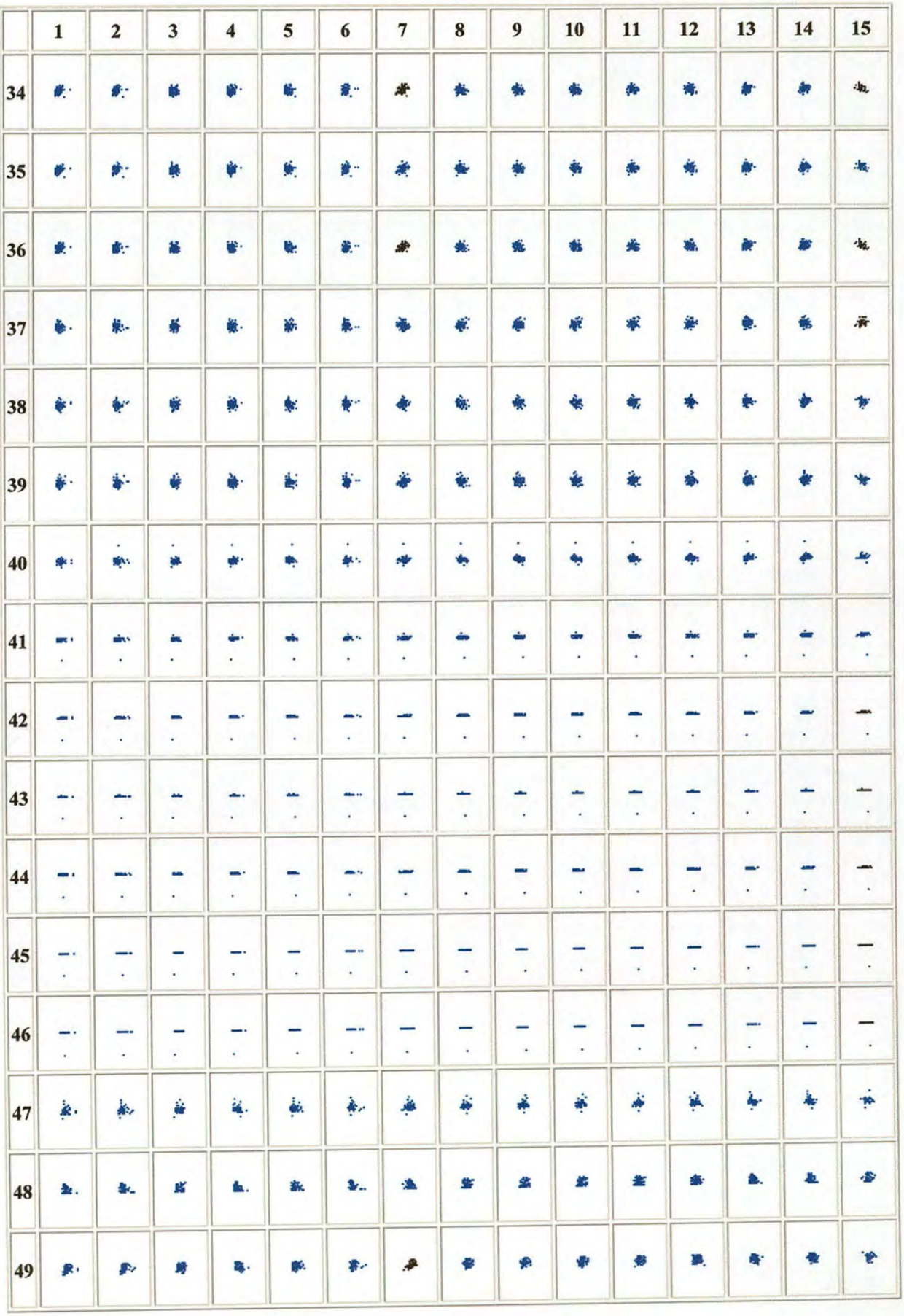

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
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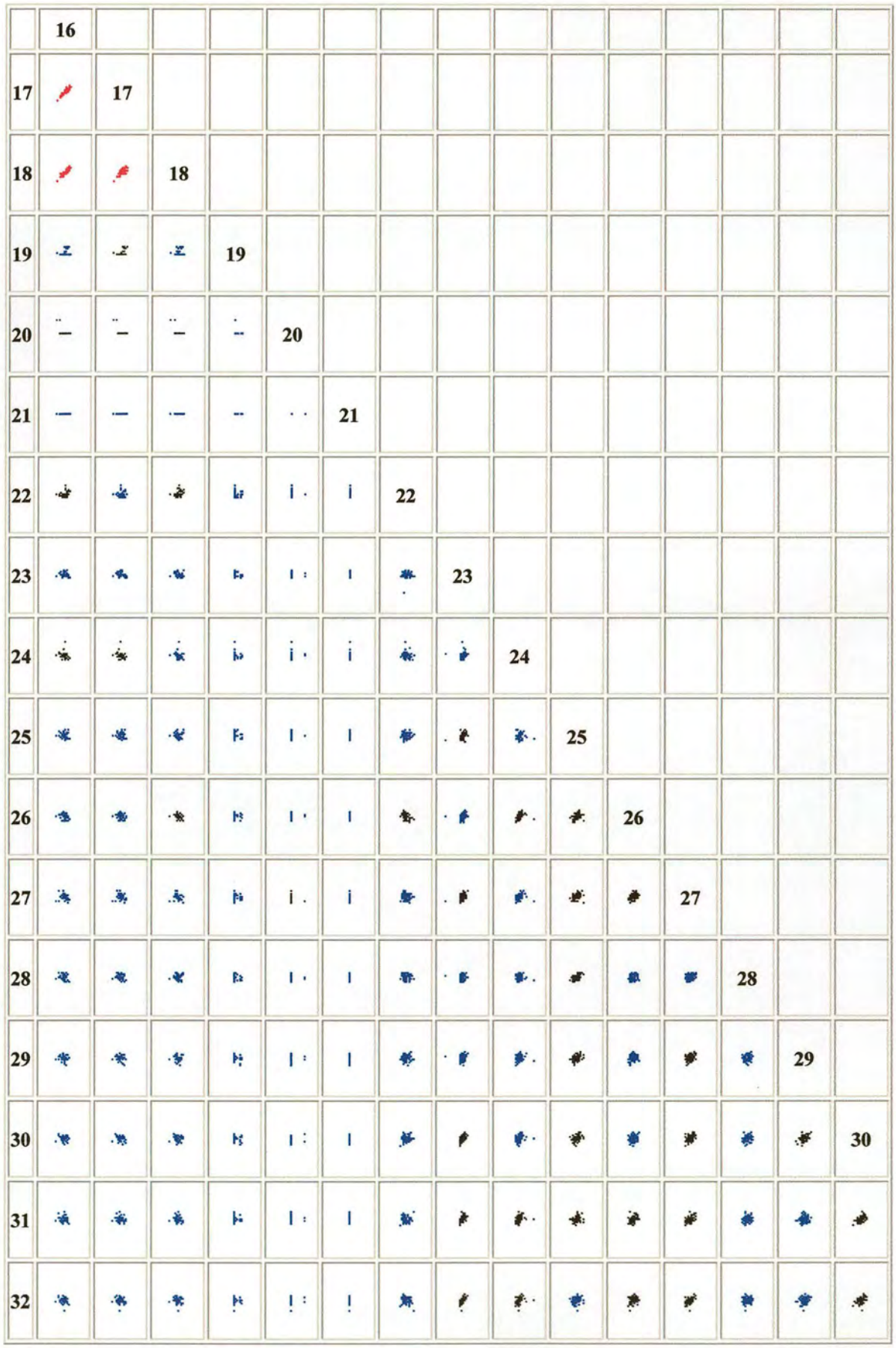
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| | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 |
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| | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 |
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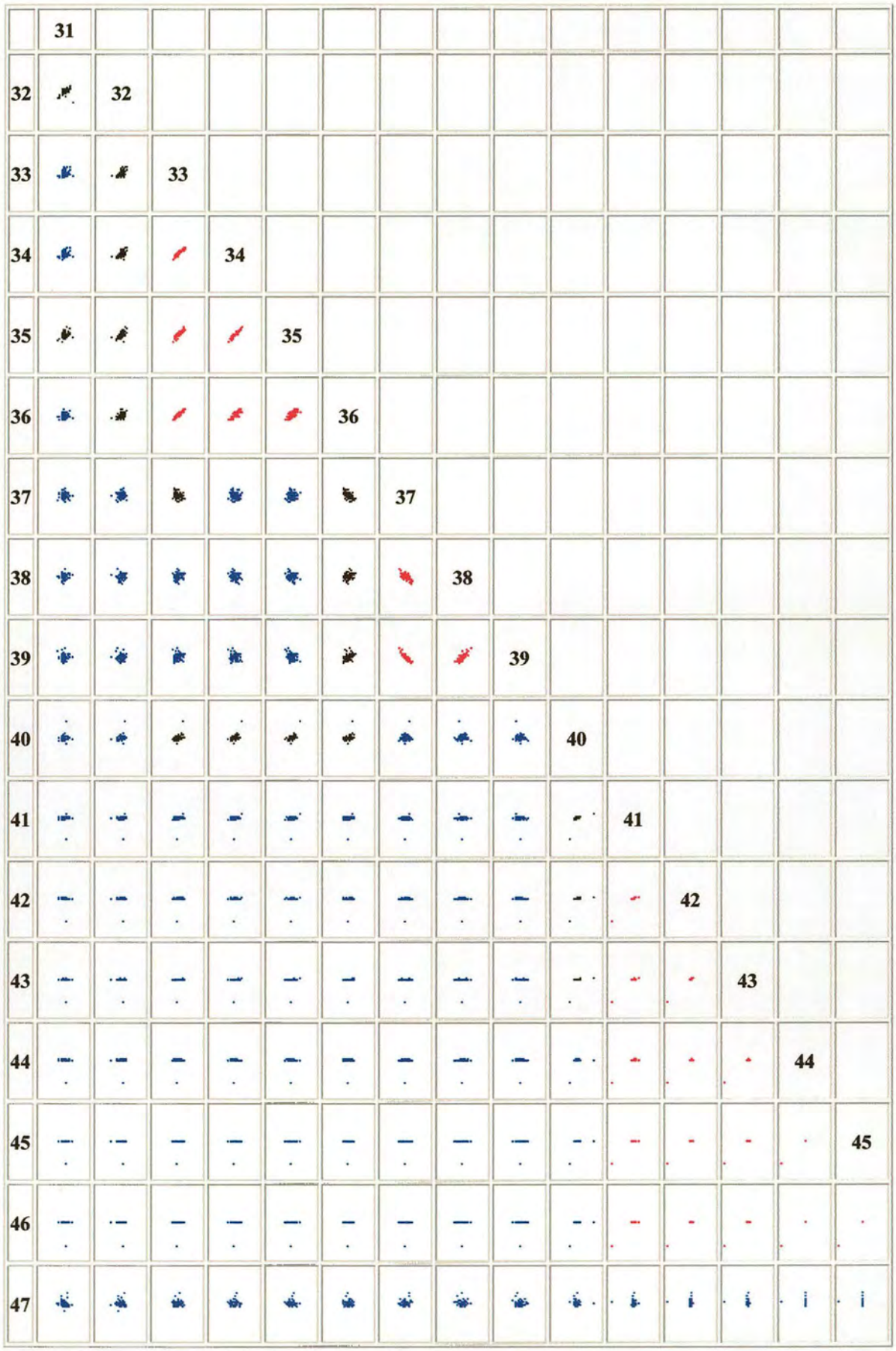
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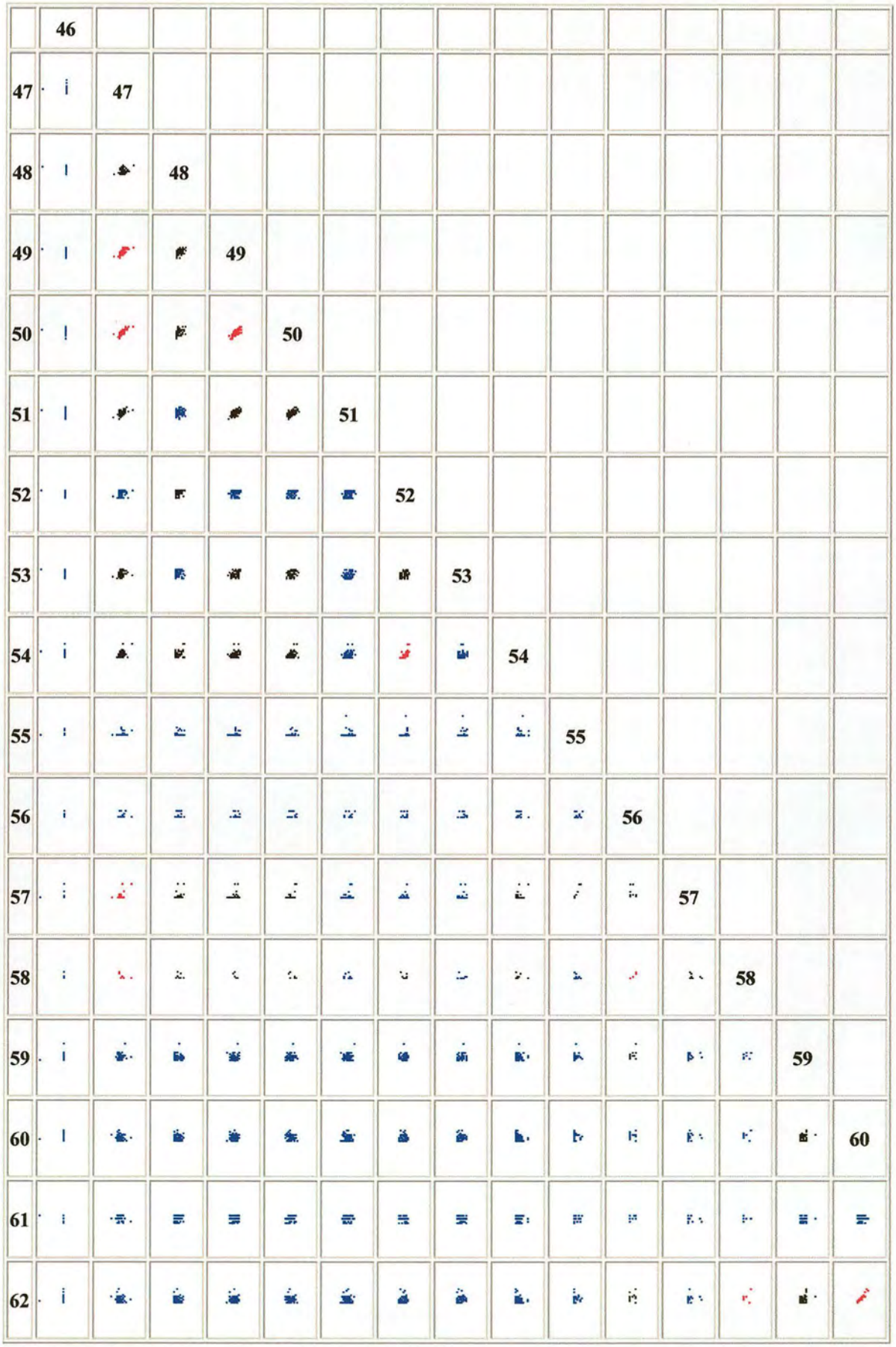
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**Appendix 4 Genotype data used for QTL mapping.** Scoring code: **a**: both alleles homozygote to majus. **b**: both alleles homozygote to molle. **c**: one allele heterozygote to molle. **d**: one allele heterozygote to majus. **h**: both alleles are heterozygote to each other. **-**: missing data.

**Population name: F2 JI98 x molle**
**Population: F2**
**Number of loc: 197**
**Number of individual: 107**

**11CACJ098 ; 1**
b-bah bhhbb hbhbh habha bhbdb bhhhh ddhbb hhbhb hhhhh bhhhh h-db- hahbd hbhbd bdbbb bhbdd babhb -bhhh bbha- -bb-h db-bh bbhbb hb
**STY ; 2**
bhbah bhhbb hb-bh -abba bbbhb bhhhh ahabb hbhhh -hhhh bhbbh hhhb- hahbh bbhbh bhbah bhbhh babhb -bahh bhhah hbb-h hb-bh hbhhb bb
**12AGCJ213 ; 3**
b--dd bb-bb dbdbb -ddbd bb--d bdddd dddbb ddbbd ddbdd bdbbd -dbd- dddbd dbdbd bdddd bdbdd ddddd -dddd bddd- -bb-d db-dd bdddd -b
**11AGGJ135 ; 4**
b-bah bhhbb hbhbh babbd bbbhh bhhhh ahhbb hbbbh hhbhh bhbbh bhbb- hhhbh bbhbh bhbhh bdbhh habhb -bhhh bbhab bbb-h hb-bh abddb bb
**11AGGJ177 ; 5**
b-bah bhhbb hbhbh babbd bbbhh bhhhh ahhbb hbbbh hhbhh bhbbh bhbb- hhhbh bbhbh bhbhh bhbhh dabhb -bhhh bbhab -bb-h hb-bd dhhhb bb
**11CATJ091 ; 6**
b-bah bhhbb dbhbb haabd bbhh- bhhhh hahbb hhbbh habah bhbbd -hb-- addb- dbdbd bdd-b bdbhh -ahdb -bddd bdbab -bh-h db-bh adhhb -b
**11CATM165 ; 7**
b-bah bhhbb hbhbb haach bbhh- bchcc cahbb hcbbh cacah bhbbh -cb-- ahhb- hbhbh bhh-b bhbhh -ahhc -bhhh bhbab -bb-c hb-bc achcb -b
**11CATJ315 ; 8**
b-bah bhhbb bbhbb haabd bbhhh bdhdh hdhbb hhbbh habah bhbbh b-bh- dhhba hbhbh bhdba bhbhh hahdb -bhdd bhbd- -bb-h hb-bh ahddb bb
**ZS046 ; 9**
bhbah bhhbb bb-bb -aab- bbhhh bhhhh hahbb hhbbh -abah bhb-h bhbh- ahhba -bhbh bhhha bh-hh hahhb -bhhh bhbah hbb-h hb-bh ahhhb bb
**11CACM181 ; 10**
b-bah bhhbb hbhcc haabc bbhch bhhhh -ahcb hhbch hacah bhcbh b-bh- ahhba hbhbh bhcha bhbhh hahhb -bhhc chca- -bb-h hc-ch ahccb cc
**11AGGM412 ; 11**
b-bah bhhbb cchbb -a--- ----- ----- --h-- ----- ----- --bch bhbh- ahhba hbhbh bchha bh--- ----- -bhhh bhbab -bb-h hb-cc ahhhb bb
**11AGGM176 ; 12**
b-bah bhhbb cbhbc hacbh bbhhh bhhhh hhcbb hbbbh habah bhbbh bhbc- ahhba cbhbh bhhha chbhh hachb -bhhh bhbab abb-h hb-bh ahhhc cc
**MS10 ; 13**
-hbah bhhbb hbhbh babbb -bhhh bhhhh aacbb hbbbh habah bhbbh bhbbb ahhbh bbhb- bhbah bh-hh habhb bbhhh bbhab hb--h h---- ----- --
**14AGCM226 ; 14**
a---h ccccb cbach aa-ba bbhcc bchhh hhcca ccccc ccccc chcbh bhhb- hhcbc cchch ccchh bcbch hhbcb -chcc bcccc -cb-c ha-ch abhhb hb
**12ACAM283 ; 15**
a-b-c bhhcb aahba aaa-a cchh- bhhhh hahba hhbbh habah cacch bccb- ahhba hchbh chbha -hcca habhh -chhh chcab -cc-h h--ch aha-h -a
**11CACM935 ; 16**
a--bh chhbb aahba aaaca bbhhh bhhhc chhba hbcbh hhbch bhbbc b-hb- hccbc cbhbh bcbhh bhchh chbhb --hcc bbch- -bb-h ca-ch bhahc ha
**11AGGM178 ; 17**
a-ccc ccccc aacca aaaca ccccc ccccc cccca ccccc ccccc ccccc cccc- ccccc

ccccc ccccc ccccc ccccc -cccc ccccc -cc-c ca-cc ccacc ca
**12AGCM156 ; 18**
a--cc cc-cc aacca aaaca -cccc ccccc cccca ccccc ccccc ccccc cccc- ccccc
cccch ccc-a c-cch ccccc -cccc ccccc -cc-c ca-cc accah -a
**11CATM338 ; 19**
a-bah bhhhb aahba aaaha chhh- bhhhh aahba abbba habah cacbh -hh-- aaab-
c-hca hab-- hhbha hachc -chhh chhac -hc-h ha--h acaah -a
**12AGCM216 ; 20**
---cc cc-cc aacca a-aca -c--c ccccc cccca ccccc caccc ccccc cc-c- acccc
cccca cccca ccccc ccccc -cccc ccccc -cc-c -a-cc accaa -a
**11AGAM132 ; 21**
a---c ccccc -aacc aaaca ccccc ccccc cccca ccccc caccc cac-c cccc- ccccc
cc-cc ccccc cccc- ccccc -cccc -cccc -cc-c ca-cc accaa -a
**11AGAM134 ; 22**
a---c ccccc -aacc aaaca ccccc ccccc cccca ccccc caccc caccc ccc-- cc-cc
ccccc ccccc cccc- ccccc -cccc -cccc -cc-c c--cc accaa -a
**14AGCM086 ; 23**
a---h bh-ch caacc aaaca cchch bchhh hhcca ccccc chccc chccc chhb- chhbc
chcbh ccchh bcchh ccccc -cchc ccccc -hc-c ha-ac accaa -a
**14AGCM238 ; 24**
a--cc cc-cc aacca a-aca ccaac ccccc cccca ccccc ccccc caccc cccc- ccccc
cccca cccca ccccc ccaca -aaaa ----- --a-c ca-cc acaaa -a
**11AGAM342 ; 25**
b---h chhha aabhh habhc chhbb cacba cchhc ahbaa baaab ccbha haah- hbcha
hhhah hbbhh acbb- bhhhh -hhha -hahh -bh-a ba-aa aabhh -h
**11AGAM056 ; 26**
c---c cccc- ccccc caccc ccccc ccccc acccc accca caacc cc-ca aacc- cbcca
cccac cccac a-cc- ccacc -cccc -caca -cc-a ca-aa aaccc ca
**12AGCJ282 ; 27**
b--dd bb-dd ddddd dbbdd bdbbd dddbd dbbdd d-bbd ddddd dbbdd ddbd- ddddd
ddddd ddddb bdbbb bddd- -bd-b dddbd -d--d bd-dd ddbdd -d
**M291 ; 28**
c---a cccca ccccc aaccc ccccc ccccc acccc accca caacc cccaa accc- cbacc
accac ccccc cccc- cccac -cccc -cc-c -cc-c ca-ca aacca -a
**ZS018 ; 29**
bhhha hbbaa hh-hh -abbh bhhhh hhabh ahb-h ahbha -aahb hbbha -ahh- hb-ha
-hhah hhhah ahbbb hhaah -hhhb ahaba hhh-- ba-aa ahhha ha
**11AGGM091 ; 30**
b-hhh cbbba hhhhh aabhc bhchh hhhbh acbhh ahbha haahc cbbha ahbh- hbhhh
hhhah hchhb hcbbb hhahh -bhhb ahaca hhh-a ca-aa ahhha ha
**11AGAM243 ; 31**
c---a hbbba -ccch aabhh bhhhh hhhbb ahbhc ahbca haacc cbbaa acbc- hhacc
accah ccccb chbb- chaac -bacb -aaba -cc-a ba-aa aahcc -a
**11AGAJ245 ; 32**
b---d hbbbd hdddh ddbhh bhhhh hddbd ahbhh dhbha haadh dbbda adbh- hhdhh
dhhdh hhhhb hhbb- hhddh -bdhb -ddbd -hh-a ba-dd adhh- -d
**11AGAM268 ; 33**
b---a cbcba hcchc aabhh chhhh hchbc ahchh ahbha caach cbcaa acbc- hhahc
ahhah hhchb hhbb- hhaah -bahb aaaca -hh-a ca-aa aahhh -a
**SQUA ; 34**
bahaa hbbaa hh-ha -abhh bhhhh hhhbh ahbhh ahbha -aahh hbbhh ahbh- hhahh
ahhah hhhhh hhbbb hhaah -bhhb aaaba hhh-a ba-aa ahhha ha
**12AGCJ195 ; 35**
b--dd d--dd ddddd ddbdd bdddd dddbd ddbdd ddbdd ddddd dbbdd ddbd- ddddd
ddddd ddddb ddbbb ddddd -bddb dddbd -dd-d bd-dd ddddd -d
**11CATJ316 ; 36**
b-haa hbbaa hhhha aabhh bhhh- hhdbh ahbhd ahbhd haahh hbbaa -hb-- hddh-
a-had dhh-d dhbbb hhaah -bahb aaabd -hh-a ba-aa ahhha -a
**11CATJ345 ; 37**
b-haa hbbaa hhhha aabhh bhhh- hhdbh ahbhd ahbha haahh hbbaa -hb-- hhah-
a-hah hhh-h hhbbb hhaah -bahb aaaba -hh-a ba-da ahhha -a

**11AGGJ219 ; 38**
b-hda -bbaa hhhha aabhh bhhhh hhhbh ahbhh dhbha haahh hbbaa ahbh- hhahh
dhhah hhhhb hhbbb hhaah -bahb aaaba -hh-a ba-aa ahhhd dd
**11AGGJ110 ; 39**
b-haa hbbaa hhhha aabhh bhhhh hhhbh ahbhh ahbha haahh hbbaa ahbh- hhahh
dhhah hhhhb hhbbb hhaah -bahb aaaba -hh-d ba-aa dhhhd dd
**11CATM351 ; 40**
b-haa hbbaa hchha aabhh bhhh- hh-bh ahchh ahbha haahh hbbaa -hb-- hhah-
a-hac ch--c ccbcb hcaac -bahb aaaba -hh-a ba--a accca -a
**SBP ; 41**
bahaa hbbaa hh-ha -abh- bhhhh hhhbh ahbhh ahbha -aahh hbbaa ahbh- hhahh
-hhah hhhhb hh-bb hhaah -bahb aaaba hhhaa ba-aa ahhha aa
**12ACAJ160 ; 42**
b-h-a hbbaa hhhha aab-h bhhh- hhhbh adbhd dhbha hadhh hbbaa ahbh- hhahh
ahhad hhhhb -hbbb hhaah -bahb aaabd -dh-a b--aa ahdda da
**11AGGM143 ; 43**
b-haa hccaa hhhha aabhh chhhh hhhch ahchh ahcha haahh hbcaa ahbh- hhahh
ahhah hhhhb hhbbb hhaah -bahb aaaba -hh-a ca-aa ahhha ha
**11CACM082 ; 44**
c-caa cccaa cccca aaccc ccccc ccccc acccc accca caacc cccaa a-cc- ccacc
accac ccccc -cccc ccaac -cacc aaac- -cc-a ca-aa accca aa
**12ACAJ074 ; 45**
b-hda hbbdd ddhha aab-d bhhh- hhhbh dh-hd dhb-a haahh hbbaa ahbh- hhahh
ahhad hhhdb -hbbb hhaah -hadb ddabd --d-d b--ad ddddd -d
**12ACAM072 ; 46**
b-h-a hbcaa chhha aabch bhhh- hhhbh acchh ahc-a haahh hbcaa ahbc- hhahh
ahhah hhhhb -hbbb hhaah -hacb aaaba --h-a c--ah ahcca -a
**11AGAJ325 ; 47**
b---a hbhda hddha aabhd bhhhh ---b- --bhd ahbdd haadd dbbda adbb- hhadd
adddd ddddb dhbb- dhaah -dadb -dabd -dh-a bd-dd adhha -a
**12AGCM058 ; 48**
c--aa cc-aa cccca aaccc cc--c ccccc acccc accca caacc cccaa accc- ccacc
accac ccccc ccccc ccaac -cacc aaaca -cc-a ca-aa aaccc -a
**11CATJ377 ; 49**
b-hda hbbaa hhhha adbhh bhhh- hhdbh ahhdd adbhd hdahh hbbaa -hb-- hhah-
a-hah hhh-h hhbbb hhaah -bhhb aaaha -hh-a ha-dh dahha -a
**12AGCM242 ; 50**
c--ca cc-cc cccca aaccc ccccc ccccc -hccc accca caacc ccbaa acbc- ccacc
accac ccccc chccc cca-c -cacc aaaca -cc-a ha-ac aaccc -a
**11AGGM274 ; 51**
--haa hbbaa hahha aaaha bhhhh hhhch ahhha ahbha haahh hbbaa ahbh- hhach
ahhah hhhhc hhbbb ccaah -bahb aaaha -hh-a hh-hh ahaha aa
**PLENA ; 52**
b-haa hbhaa ah-ha -abh- bhhhh hhhbh ahbh- ahbha -hhah bhhaa ahhh- hhahh
ahhah hhhhb h-bbb hhaah -aahb aaab- hhh-a bh-ah ahhha ha
**11AGAJ161 ; 53**
h---a hbbbd hdhhh aaahd hhhhh hhdbh ahdhd ahbdd hadhh hbhaa ahbd- hhahh
adhah hhdhh hbbb- hdabh -bddb -dahd -bb-a hd-hd adhdd -d
**11AGAM038 ; 54**
c---a cccca c-ccc aaccc ccccc ccccc acccc acacc ccccc cccaa ccbc- ccacc
accac ccccc cccc- cca-c -bccb -aaha -cc-a ha-cc aaccc -a
**14AGCM171 ; 55**
a---h ah-cc haahb aaaaa ccacc cccbc chhba hhahh chacc cahac abhh- hahah
bhhch hahca chhhh aaahh --aaa abhba -ca-b ba--a ahbac ha
**11CACM167 ; 56**
hachh hhabh achhb hbbab bbach hhhbh hccbh chahc haahh hchah c-cc- hhhaa
bcccc cccca chccb aaahh -haca achb- -bh-b bh-hh hhbcc hb
**214 ; 57**
hhhhh ahhbh aa-hb -bbab bbahh hhhbh hhhbh hhahh -aahh haaah ab--- -haah
hhhhb hahba hahhb aahhh -haha abhba hb--b hh-hh h---h hb
**11CACM083 ; 58**

c-ccc acccc aaccc ccccc ccacc ccccc ccccc ccacc ccaac cacaa c-cc- caccc
caccc aacaa caccc aaacc -cacc cccc- -ca-c cc-cc ccccc ac
**11CATJ109 ; 59**
d-ddb dddbd ddddb ddbdb bbdd- ddbhh ddddd dbddd ddddd ddddd -bd-- ddbd-
ddddb ddd-d ddddb ddhdd -dddb dbhbd -bd-d bd-bb dddbb -b
**12ACA?313 ; 60**
b-h-b ahhbd bbbbb bbb-b bbhd- ddbdd ddddb dddbb dhdah hahaa hdhh- dhbhh
ddbdb hddba -dhhb bbhah -hahb hhhba bha-h d--hb hdhda -a
**11CACM088 ; 61**
b-hcb ahhbh aabbb bbbhb bbhhh hhbhh hhhha hhacc hhhah hahaa h-hh- ahbhh
hhbhb haaba hahhc bahac -hahb hhcc- -ca-h bh-hb hhhbb ab
**12ACAJ313 ; 62**
b-h-b ahhbd bbbbb bbb-b bbhd- ddbdd ddddd dddbb dhdah hdhda hdhh- dhbhh
ddbdb hddbd -dhhb bbhah -hahb hhhba bda-d d--hb hd-bb -b
**11CATJ230 ; 63**
b-dhb aadbh aabbb bhbhh bbhh- ddbdd dddh- -hh-- ---ah hahaa --h-- ahbh-
h-bhb ddd-b baddb dbddd -dhhb hhbb- --a-a dh-h- dddbb -h
**11CATM099 ; 64**
b-hhb aahbh ahbbb bhchc bbhh- hhchh hhhha chhcc cccah hahha -ch-- acbh-
hcbhb haa-b baccc -bhah -hhhb hhbca --a-a hh-ac hahbb -h
**11AGGM061 ; 65**
c-hhb aahbh ahcbc bhbhc bbchh chbch hhhha hchcb hhhah hahha hchh- ahbhh
hhchb haaba bahhb cchah -hhhb hccba -ha-h hh-cb hhhbb ah
**ZS042 ; 66**
bahhb aahbh ah-ba -hbhh bbhhh hhb-h hhhha hhhbb -hhah hahha hhhh- ahbhh
-hbhb h-aba ba-hb bbhah -hhhb hhhba haa-a hh-hb hahb- --
**12ACAJ215 ; 67**
b-h-b aahbh ahbbh bhb-h bbhh- hhbdh hdhha hhhbb hhhah hahda hhhh- ahbhh
hhbhb hadba bdhhb bbhdh -hhdb hhhbd -dd-d hh-hb dahbb -h
**11AGGJ108 ; 68**
b-hhb aahbh ahbbh bhbhh bbbhb hhbhh hhhha hhhbb hhhah hahha hhhh- ahbhh
hhbhb haaba bahhb bbhah -hhhb hhhba -aa-d hh-hb dahbb ah
**11AGGJ222 ; 69**
b-dhb aadbd dhbbh bhbhh bbbhb hhbhh hhhhd hhhbb hhhah hahha dhhh- ahbhh
hhbhb haaba baddb bbhah -dhhb hhdba -aa-a hh-hb dahbb ah
**12ACAJ427 ; 70**
b-h-b ddhbh ahbbd bhb-h bbbh- hdbdd hdhha hhhbb hdhah hahda hhdd- ahbhh
hhbhb haaba -ahhb bbhah -hddb hhhba -aa-a h--hb daabb -h
**12AGCJ182 ; 71**
b--d- dd-dd ddbbd bdbdd bbbdb ddbdd ddddd dd-bb ddddd ddddd dddd- ddbdd
ddbdb dddbd bdddb bbddd -dddb dddbd -dd-d dd-db bddbb -d
**12AGCM187 ; 72**
c--cc aa-cc acccc ccccc ccccc ccccc cccca ccccc cccac cacca cccc- acccc
ccccc caaba baccc cccac -cccc cccca -aa-- cc-cc caacc -c
**11AGGJ227 ; 73**
b-dhh aadbh ahhbh bdbhd bbhhh hdhhd hhhha hhhhb hhdah hahha hhhh- ahbhh
hhbhb haaba bahhb bbhah -hhhb hhh-a -aa-d hh-hb hdabb dh
**14AGCJ113 ; 74**
b---b adahb hadbb bdbhd bbdhb ddbdd ddhha hhhbb hhhah hahha hhhh- ahbhd
hdbhb daaba bahhb dbhah -hdhb hhbbd -aa-a hd-db bdddb ad
**14AGCM248 ; 75**
b---h aaahb hacbb bhbhc bbchh hcbhh hhhha hhhcb hhhah hahha hhhh- ahbhh
hhbhc haaba cahhb bhhah -hh-c hhhca -aa-a hh-hc caahb a-
**14AGCM123 ; 76**
a---h aaahb haabb bhbhh bbcch hcbhh hhhha cchbb hh-ah hahca hccc- acbch
ccbac caaba bahhc bchah -hchb hchba -aa-a ha-cb aaaac aa
**11AGAM293 ; 77**
b---c aaahb hacbc bhchh bbhhh hcbhc cchha -hhbb hhh-h haaaa hhhh- ahcha
hhcah haaca cahh- hhhah -hhhb -chba -ab-a hh-hh baahb -h
**12ACA/293 ; 78**
b---c aaaha aacbc bhcah bbhhh hcbac ccaaa aaaba hahaa haaaa hhhh- ahcha

hhcah haaca cahh- hhhah -hhhb -chba -ab-a hh-hh baahb -h
**11AGGJ360 ; 79**
d-dbd ddddd ddddd dbbdb ddddd dbddb ddddb d--dd dddbd ddddd dddd- ddddd
ddddb ddbbd ddbbd dbddd -dbbd ddbdb -dd-b dd-bb dbddb dd
**ASYN ; 80**
hhhbh ahhaa -hhhh ---a- ---h- hbbhb ha--- abbhh hahbh hhaaa --hh- -hhah
ahaab habbh ah-bh hbhha -hbbh hhbhb bah-b h--h- -bhab ah
**11CACM133 ; 81**
a-cbh ahhcc ahacc hcchc hchha bbbhb hhhcc habha chhha hhhhh c-hh- hhhch
hhhhb hhaca chbba hbcha -cbbh haha- -bh-c ah-hh bchbb ch
**11AGGJ152 ; 82**
d-hbh ahhhh dahhb hbbab hhhhd bbbhb hddhb aabbd hdhhh hbada hhhh- hhhhd
hahab hhabh hhbba hbhha -hbbh bhhaa -hh-h ah-hh bbhah db
**14AGCM142 ; 83**
a---c a--cc cahcc aaaab cccch bbcbb aaacc a-bba caccc ccaaa ccch- hchcc
cacah chach cccca hbahb -hbbb cccac -ch-c aa-cc abcaa ha
**11AGAJ185 ; 84**
b-dbd dddbd bbddb bbbdb dbdbd bbdbb ddddb ddbbd bdddd ddddd d-bd- dbddd
dddd- bddbd ddbbd ddddd -dbbb bddd- -dd-d db-dd bdbbb bb
**11CACM185 ; 85**
b-hbh ahhhh hbhhb bbbdb hbhbh bbhbb ahahb aabba bhhhh hhdaa h-bh- hbhah
ahhah bhdbh dhbbh hhaha -hbbb bhha- -ah-h ah-hh bbbhh hb
**11AGAM354 ; 86**
a---h ahhhh hahhh bhbah hchba cccbc aaahb aacca cahha ccaaa chch- abahc
cacac haabh hhbb- ahaha -hbbb -aaaa -hh-h ah-hh hccha -c
**14AGCJ122 ; 87**
a---h dh-hb hadhh bhhad hbhba bbhbb aadhh aabba bahha hhaaa hhbh- dbdhh
hahad bhdbh hhbba hhaha -hbbb hhhad -hh-h dd-hh dbhhh db
**14AGCJ062 ; 88**
a---h dh-hb hdhhh bhhah hbhba bbhbb aadhh ddbba bdhha hh-aa hhbh- ddddd
dahah bddbd dhbba ddddd -dbbb bh--d -hd-d dd-dd d-dad db
**14AGCJ187 ; 89**
a---h dhhhb hahhh bdddd hbhba bbhbb aaahh aabba bahhd hbaaa hh-h- ahhhh
hahdb hhaba hhbba hdahd -hbbh bhhaa -hh-h ah-hh hbhah bb
**14AGCM038 ; 90**
a---c ac-cb hachc bccac cbcba bccbb aaacc aacca cacca cc-aa ccbc- acccc
cacab hcaca ccbba caaca -cbbc bccaa -cc-c ac-cc cccac cb
**14AGCJ273 ; 91**
a---h dhhhh hadhh hdddd hbhba bbhbb aadhh dabba bahha hbaaa hhbh- ahhhh
hahdb hhaba hhbba hadhd -hbbh bhhad -d--h ah-hh hbhah bb
**11AGAJ276 ; 92**
a---h dhhhh hahhh bhhah hbhbd bbdbh aaahh ddbbd bahha hbdda hhbh- dhhhh
hahab bhaba hhbb- hdahd -hbbh -hhdd -hd-h ah-hh hbhah -b
**12AGCM069 ; 93**
a--cc ac-cc acccc cccac cccca ccccc aaacc aacca cacca ccaaa cccc- ccccc
cacac ccaca cccca c--c- ----- ----- ----c ac--c cccac -c
**12ACAM344 ; 94**
a-c-c achbh ahhha ahh-a hchb- bbhbb aaah- aabba bacch hbaaa hhbh- hhhhh
hahac chabh -hbba hhaha -hbbh bhhaa ahh-h a--hc bhahh -h
**11AGGM090 ; 95**
a-hbh ahhbh ahhhb hhhah hbhba bbhbb aaahh aabba bahhh hbaaa hhbh- hhhhh
hahab bhabh hhbba haaha -hbbh bhhaa chh-h ah-hh chach cb
**11AGGM156 ; 96**
a-hch ahhbh ahhhb hhhah hbhca bbhbb aaahh aabba bahha hbaaa hhbh- hchhh
hahab bhaba hhbba haaha -hbbh bhhaa -hh-h ah-hc chahh bb
**12ACAJ384 ; 97**
a-h-h dhhbh adhhb ddd-h hbdb- bbhbb aadhd ddbba bahha hbdaa hhbd- hhdhh
hahab bhaba -hbba haaha -hbbh bhhad -hh-h a--hh bhadd -b
**INA ; 98**
ahhbh ahhbh ah-hb -hha- hbhba bbbbb aaahh aabba -ahha hbaaa hhbh- ahhhh
hahab haaba hhbba haaha -bbbh hbhaa ahh-h ah-hh bahah bb

**CDC2C ; 99**
dhhbd ahhbh ah-db -hhad dbdbd bbdbb aaahh aabbd -addd ddaaa dhba- hbhhh
-ahad hhhbd ddbba hddaa -hbbb ddhd- aah-d dh-ha bdddd db
**11CACJ202 ; 100**
a-hbd ahhbh ddhdb dhhah hbhbh bbhbb aaahh dabba bahhh hhdaa h-bd- hbhhh
aahah bhhbh hhbba hhhaa -hbbb dbha- -ah-h dh-hh bhhdd hb
**11CACM288 ; 101**
a-hbh ahhbh achhb ahhah hchhh bbhbb aaahh aabba bahhh hhhaa h-ba- hchhh
aahah bhhbh hhbba hhhaa -hbbb hbha- -ah-h ah-hh bhhhh hc
**12AGCM289 ; 102**
a--cc ac-cc acccc accac ccccc ccccc aaacc aacca caccc ccaaa ccca- ccccc
aacac ccacc cccca cccaa -cccc cccaa -ac-c ac-cc accac -c
**CH80 ; 103**
ahhba ahhbh ahhhb -hhaa hbhhh bbhbb aahha aabab -hhhh hhhaa ahba- hbhhh
aahah hhhbh hhbba hhhaa -hbbb hbhaa aah-h aabah bhhhh hb
**11CACM218 ; 104**
a-bbc chhbh ahhhc ahhah hbhhh bbhbb aaahh aahhh caahh hhhaa h-ba- hbhhh
aahah bhhbh hhbha hhhaa -bbbb hbha- -ah-h ah-hh bhhbh hb
**11CACJ166 ; 105**
a-bbh hhhbh ahbhb ahhah hbhhh bbhbb daahh dahhh baahh hhhaa h-ba- hbhhh
aahah bhhbh hhbha hhhaa -bbbb hbhd- -ah-h ah-hh bhhbh hb
**11CATJ180 ; 106**
a-bbh hhhhh ahbhb ahdab bbhh- bbdbb aaahd dahdd baahh hhhaa bdb-- hbhh-
addad bdd-d dhbda dhhaa -bbbb dbhdd -ah-h ah-hh bddbd -b
**12ACAJ125 ; 107**
a-b-h achhc aabha aaaaa cbhc- bcccb aaaca aah-a caccc ccaaa acba- abhch
aaaaa ccaca -cbha ccaaa -bccc hbaaa --c-c a--cc bcaac -a
**11CACM103 ; 108**
c-ccc ccccc acccc acccc ccccc ccccc aaacc caccc acacc cccaa c-ca- cccca
aacac ccccc cccca cccaa -cccc cccc- -ac-c ac-ac ccccc cc
**11CACM292 ; 109**
b-hhb hhhhh hhccc hhhhh -hcc- a-cca hcahh hhccc hhccb chahh -cb-- ahhh-
a-hhh hbb-c cahah chhbh -ahhh hbhhc -hb-h hh-cc hhhaa -h
**11CATJ281 ; 110**
abhhh hhhha hhhhd hhhhd ahhh- dadha addad dhhbb aahhb hdadd -dd-- ddhh-
d-haa hbb-h hddah dhabh -aaah abhhb -dh-a hd-hh hhhaa -h
**11AGGJ103 ; 111**
a-dhh hddha bbddh hhhdh abdhd ddbdd adaah hdhbh ddbdd ddadh hbhh- ahhdb
dabaa bbbhh ddhdb ddabh -addh d-hhh -hd-d hh-hh hdhdd db
**ZS113 ; 112**
aahhb hhhha bb-hh -hhhh abhha aabha ahaah h-hbb -abhb hhahh ab-h- ahhhh
aabaa hbbhh hhhab hhabb -aaah abhhh hhh-a bh-hh aa-aa ab
**12ACAM306 ; 113**
a-h-c hhhba bbhhc hcc-c abhh- aacha ahaac hchcb aabhb hhahh hbhha ahchb
aabaa hbbhh -ahab hhabb hhaah abhhb -ah-a b--hh ahcaa -b
**ZS048 ; 114**
aahhb hhhba -b-hh -hhh- abhhh aabha -haah hbhbb -ahhb hhahh hbhh- ahhhb
-abaa hbbhh ha-ab hhabb -aaah abhhb hah-a bh-hh aahaa ab
**11AGGJ240 ; 115**
a-h-b hhhba bbhhc ccc-a abhh- aabha ahaah hbhbb aabhb hhach hbcc- ahhhb
aabaa bbbhh -ahab hhabb -aaah dbhhb -ah-d b--hh dahaa -b
**12ACAJ179 ; 116**
a-h-b hhhba bbhhd ddd-d dbhh- aabha adddd hbhbb aabhb hhahh hbhh- ahhhb
aabaa bbbhh -dhab hhdbb -daah dbhhb --h-a b--hh aahaa -b
**12ACAM177 ; 117**
a-h-b hhhba bbhhh hhh-h abhh- aabha ahaah hchcb aabhb hhahh hbhh- ahhhb
aabaa bbbhh -ahab hhacb -aaah abhhc --h-a b--hh aahaa -b
**12AGCJ096 ; 118**
a--db dd-bd bbddd ddddd dbddd ddbdd ddddd dbdbb ddbdb ddddd dbdd- ddddb
ddbdd bbbdd ddddb dddbb -dddd dbddb -dd-d bd-dd ddddd -b
**11CACM265 ; 119**

a-hcb chhba bbhhh hcchc abhhh aabha ahaah hbhcb aachb hhahh h-hh- ahhhb
aabaa bbbhc hahab hhabb -aaah achh- -ah-a bh-hh aahaa ac
**11AGGM240 ; 120**
a-hhb hhhba bbhhh hchhh achhh aabha ahaah hbhbb aabhb hhahh hchh- ahhhb
aabaa bbbhh hahab hhabb -aaah abhhb -ah-a ch-hc aahaa ab
**12ACAM286 ; 121**
a-c-b cccbb bbccc ccc-c accc- aacca acaac ccccc aabhb hhahh hbhh- ahhhb
aabaa bbbhh -ahab hhabb -aaah abhhb -ah-a b--hh aacaa -b
**14AGCJ092 ; 122**
a---b hh-hb dbbhh dhhhd abhhh aabda adddh hbhbb ddbhb hhadh hbhh- ahhhb
aabaa bbbhh hdhab hhabb -aadh dbhhb -dh-d bh-hh bdhha ab
**14AGCM314 ; 123**
a---b hhhhb abchh hhhhc abhch aabha ahaah hchcb aabhb hhahh hbhh- ahhhb
aabaa bbchh hahab hhacc -aaah abhhc -aa-a bh-hh hhaha ac
**14AGCM269 ; 124**
a---b hhchb aaabh aaaha abhcc aabca ahaaa hbhbb aabcb hhahh hbhh- ahhhb
aabaa bbcch cacac chabc -aaah achhb -a--a ca-hc ahhaa aa
**12ACAM128 ; 125**
a-a-c acacc aacca aaaca aacc- aaaaa caaca aacac ccacc caacc aacc- accac
acccc cacaa -aaaa caccc -acca cccaa --c-c a--cc cc-aa -a
**12ACAM191 ; 126**
a-aac acacc aacca aaa-a aacc- aaaaa caaca aacac ccacc caacc aacc- accac
acccc cacaa -aaaa caccc -acca cccaa -cc-c a--ca ccaaa -a
**12ACAM122 ; 127**
a-a-c -c-cc aaccc aac-c aacc- aaaac caaca aac-c ccacc caacc aacc- accac
acccc cacaa -aaaa caccc -acca cccaa -cc-c a--ca cchaa -h
**12ACAM256 ; 128**
a-a-c acahh aahhh aac-c aacc- aaaaa caaca aacac ccacc caacc aacc- accac
accch cacaa -aaaa caccc -acca cccaa -cc-c a--ca cccaa -c
**11AGGM105 ; 129**
a-aah ahahh aahhh aahhh aahha aaaaa haaha aahah hhahh haaah aahh- ahhah
ahhhh hahaa haaaa hahhh -ahha hhhaa -hh-h ah-ha hhhaa ah
**11CACM275 ; 130**
a-aac acach aahch aahhc aacca aaaaa caaca aacac ccacc caahh a-hh- ahhac
ahhhh hahha haaaa hahch -ahba hhha- -ch-h ah-ha hhhaa ah
**11CATM155 ; 131**
a-aac acacc aaccc aaccc aacc- aaaaa caaca aacac ccacc caacc -ac-- acca-
acccc cac-c caaaa caccc -acca cccaa -cc-c ac-ca cccaa -c
**CYC ; 132**
aaaah ahahh aa-hh -ahhh aahha aaaaa haaha aahah -hahh haahh aaha- ahhah
-hhhh hahaa ha-aa hahhh -ah-a hhhaa hhh-h ah-ha hhhaa ah
**12ACAM310 ; 133**
a-a-c acacc aaccc aac-c aacc- aaaaa caaca aacac ccacc caacc aaca- acccc
acccc cacaa -aaaa caccc -acca cccaa -cc-c a--ca cccaa -c
**11AGGJ390 ; 134**
a-dhb dhhbd aahhh haahh aaahh ahaha ha-ha aahbd ddhhh aaahh dabh- aaahb
abhab hahah ddaah bahah -abba hbhah -ha-a ah-hd hhdda bb
**14AGCM386 ; 135**
a-hhb hhhbh aahhh haahh aaahh aha-a -aa-a aaccc ccccc aaahh chbh- aaahb
abhab hahah ccaah bahah -abba hbhah -ca-a ah-hb hhcca bb
**12AGCJ361 ; 136**
a--dh hb-hd aadhd hbbbh aabbh ahaha daahd bahhh hbhbh aaahb hdhh- daadh
ahhah hahdd baadd ---a- ----- ----- ----- ah--d ----- --
**203 ; 137**
ahhhb hbhbh -a-hh -aahh aaahh ahaha aa-ha aahbh -hhhh aaaaa hhbh- aaabb
-bhah hahab hh-ah ba-ah -abba bbhhh hha-a ah-ha hhhhh bb
**11CACM222 ; 138**
a-hhb cbhbh haaha haahc aaahc ahaha aaahh ahhch hchch aaaaa h-bh- haabb
hbahh ahaab acahh bahab -abba cbhh- -ca-a ah-ha hhhch cc
**11AGGJ170 ; 139**
a-hhb hbhbh hahha haahh aaahb ahaha dadhh ahhbh hhhhh aaaaa hhbh- hdabb

dbhah ahhab dhaah bahab -abba bbhhh -ha-d dh-ha hhhhh bb
**11AGGM169 ; 140**
a-hhb hbhbh hahha haahh aaahb ahaha haahh ahhbh hhhhh aaaaa hhbh- haabb
abhah ahhab ahaah bahab -abba bbhhh -ha-a ah-ha hhhhh bb
**11AGGM125 ; 141**
a-chb hbhbh hahha caahc aaacb ahaha aaabh achbh hhhhc aaaaa hhbh- haabb
accah hhhhb acaah baaab -acca cchhh aha-a ah-ha hhhhh bb
**14AGCJ210 ; 142**
a---d bbdhh hahhb hbbbb dabhh bdaah aada- hhhhd bhhhb h-ddh bahd- hbbbh
bddhh dbhdb dbaaa ahahh -hbhd hbhdb -ba-d hb-hh hdbhh hh
**12ACAJ26 ; 143**
a-h-d bbdhd -bdbb bbb-b aabh- bhaah addab hhdhh bhhdb hhhah bahd- hbbbh
bahhh hbhhb -baaa ahahh -bbha hbhhb -bb-a h--hh dbbhh -b
**11CACJ111 ; 144**
a-hha bbhhh adhbd ddaba adbhh bdadh daaah hhhhh bhhhb hdhah b-hd- hbhbh
bahhh hbhhb bhaaa dhahh -bhha bddh- -hb-b ha-hb hhddh hh
**12ACAM132 ; 145**
a-h-a bbhhh --hbb aca-a aabh- bhaah aaaac hhhhh bchhb hahah baha- hbbch
bahhc hbhhb -baaa ahahh -hbha hahhb -cb-a b--hh hbahh -h
**11AGGJ124 ; 146**
a-hha bbhhh aahbb ahaba aabhh bhddh aaddh hhhhh bhhhb hadah bdha- hbbbh
bahhh hbhhb hbaaa ahahh -hbha hahhh bhb-a ba-bd hbadh hh
**11CACM343 ; 147**
a-hhh bbhch aahbh acaca aabch bhaah aahah hhhhh bhahb hahah b-ha- hbbbh
bhhhh hbhhb hbaaa ahhhh -bbha hbah- -bb-a ha-hh hbacc hh
**11CACM180 ; 148**
a-hch bbhch aahbh ahaba aabch bcaah aahah hhhhh bhahc hahah b-ha- hccbh
chhhh hbhhb hbaaa ahhch -bbha hbah- -bb-a ha-hh hcahh hh
**MS1 ; 149**
ahhhh bbhhh aahbh -haba ---hh bhaah aa-ah hhhhh bhahb hahah bahaa hbbbh
bhhhh hbhhb hbaaa ahddh cccha hbahb bbbha ha-hh hbahh hh
**11AGGJ116 ; 150**
a-hhh bbhhh aahbh adaba aabhh bhaah aahah hhhhh bhahb hahah bdha- hbbbh
bhhhh hbhhb hbaaa ahhhh -bbha hbahb bbb-d ha-hh hbahh hh
**ANT ; 151**
ahhhh bbhhh aa-bh -haba aabhh bhaah aahah hhhhh -hahb hahah baha- hbbbh
bhhhh hbhhb hbaaa ahhhh -bbha hbahb bbb-a ha-hh hbahh hh
**11AGGJ280 ; 152**
a-hhh bbhhh aahbh adaba adbhh bhaah adhdh hhhhh bhahb hahdh bdha- hbbbh
bhhhh hbhhb hbaaa ahhhh -bbha hbahb -bb-d ha-hd hbdhh hh
**11CACJ363 ; 153**
a-hhh bbhhh dahbh ddaba aabhh bdddh aaddh hhhhh bhaab hahdh b-ha- hbbbh
bhhhh hbdhb hbaaa ahhhh -bbda hbdh- -bb-a ha-hh hbadd hh
**12ACAM262 ; 154**
a-h-h bbhhh aahba aaa-a aach- bhaah aahaa cccch bhacb hahah baha- hbcbh
bhhhh hbhhc -baaa achhh -bbha hbahb -cc-a h--hh hcahh -a
**14AGCM172 ; 155**
a---h cc-hh haabh acaba aabch bhahh aahac hhhhh bhahb hahah baca- abbbh
bhhhc hbchb hbaaa ahhcc -bbha hbahc -bb-a ha-hh cbhaa hh
**14AGCM041 ; 156**
a---c cc-cc caacc acaca aacch bcahh aacac cc-cc ccacc ca-ah baha- abbch
bhchc hbhhb ccaaa ahcch -bbha ccacc -cc-a ha-cc cccaa cc
**14AGCJ183 ; 157**
a---h bbdhh haahh dhaba ddbhh bdaah aahdh dhhhh hdadd hahad hada- hbbbh
bhhhh hbbhb hbaaa ahhh- -bbba hbahb bbb-a ba-hh adhad hh
**14AGCJ244 ; 158**
a---h bbdhd haahb hhaba ddbhh hdaah dahah hhhhh hhadd hahdh badd- hbbbd
hdhhd dbhhb hbaaa ahhhh -bbha hbadb -bb-d ha-hd ahhah hh
**11AGGM198 ; 159**
a-aha bbhhh aahbb hhaca aabhh caaah haaah hhhha bhhhh hahah bhha- hbhbh
bahhh hbhhb hhaha chaha -hcha aahbh -hc-a ch-bc achch hh

**FAP2** ; 160
ahaha bbhhh aa-bb -hah- aabhh baaah haaah bhhha -hhhh hahah bhaa- hbhbh
-ahhh hbhhb hh-ha hhaha -hhha aahbh bhb-a bh--h abhbh hh
**11AGAM361** ; 161
c---c ccccc -aacc ccaca aaccc ccacc a-cac ccccc ccacc cacah bahaa accch
bhh-h hbhhb chaa- -chbh -bhca -ch-h -cb-a ca-bc aacah --
**14AGCJ221** ; 162
a---h bbb-h bddhb dhaba hdbdh bdaah haadd bhhhh bdddb dahdd bddd- dbbbd
bdhdd dbhdb dbddd ddabb -b-hb abhdb -b--b bd-bb dhbdb dd
**11AGGJ139** ; 163
a-ddd bdddb dddbb bdddd ddddd bdddb ddddd ddddd ddddd ddddd bddd- dbdbd
bdddd dbddb bdddd bdddd -dddd dddbd -dd-d dd-bd dbddb dd
**KAN4** ; 164
aabah ahhhb -h-bb -hhh- hahhh baaab hhahh hhhhh -hhhh hahah bhah- ababa
bhaah abaab aaahh baaha -hhha aahbh bhh-h hh-bh abhhh hh
**150** ; 165
a---b ahcba aaacb aaaaa bhabc bccca chhca hcahh hchha aabbh hhha- haaca
ahaah hhhhc baah- hhha- -hhhh -hach -hb-h ca-h- acaaa -a
**11AGAJ089** ; 166
a---b dbbbd ddbbb bdddd bdddb bddbd dddbb dbddd ddddd ddbbb -bdd- bdddd
dddbb ddddb dddd- ddddd -dddd -ddbd -dd-d bb-dd -bdbd -d
**11AGAJ283** ; 167
a---b ahbbh dabbb bhdad bhahb bddba ddhbb hbabh bbhda aahhb dbda- baddd
adaab ddddb bdah- dhaaa -hdhh -hdbh -da-h bb-ha hbaba -b
**14AGCM203** ; 168
a---b aacch aabch bhhah bhacb bhhba hhhbb hcach cbhha aahhb hbha- bahhh
ahaab hhhhb caahb hhaaa -hhhc hhabh -ha-h bb-ha hcaca cb
**11AGAJ312** ; 169
a---b ddddd dddbb bdddd b-ddb ---b- --dbb dbdbd b-ddd ddd-- dddd- bdddd
ddddb ddddb bddd- ddbdd -dddd -ddbd -dd-d bb-dd -b--- -b
**11AGGM190** ; 170
a-hhc ahbha abbbb cchac bhahb chhba hhhbb hbabh bbhha aahhb hcha- bahhh
ahaab hhhhb caahb ahaaa -hhhh hhabh -ha-h cb-ca cacah hc
**DEF** ; 171
ahhhb ahbha -b-bb -hha- bhahb bhhba hhhbb hbabh -bhha aahhb hbha- bahhh
-haab hhhhb ba-hb hhaaa -hhhh hhabh a-a-h bb-ha babah h-
**12ACAJ134** ; 172
a-h-b ahbha abbbb hhddd bhah- bhhba hhhbb hbdbh bbhhd aahhb hbha- bahhh
dhaab hhhhb -ddhb hhadd -hhhh hhabh -dd-h b--hd babah -b
**11AGGM051** ; 173
a-hbb ahbha abbbb hchah bhaab bhhba hh-bb hbabh bbaha aahhb hbha- bahhh
ahaab hhhhb chahb hhaaa -hhhh hhhbh -ha-h bb-ha cabah bb
**?????M356** ; 174
a-h-b ahhha abbbb hhh-h bhad- bhhba hhhbb hbabh bbaha aahhb hhha- bahhh
ahaab hhhhb -hahb hhaha -hddd hhhbh -ha-h b--ha babah -b
**11AGAM408** ; 175
a---b acchh acbbc hccac chahc cccb- cchbb hcac- -chba -cchb hhbc- cahhc
achhc chbbb bach- ch-h- ---c- ----- ----- -c--h ----- --
**11CATJ286** ; 176
a-hbh ahhaa ahhab hhhah bhad- hhdba ahhha hbabh bhaha ahhdb -hb-- bdhd-
adahb ddb-b bhadb ddddd -bddd dddbh -dd-b bh-da bbbah -a
**11AGAJ236** ; 177
a---d ahhda adhad bhhdh bbddb dhaba adhda dbabh bhdha dhhhb dhba- bdhhh
ahddb hhbhb bhdd- hhhhh -bhhh -hh-h -hh-b bd-ha hbbbh -a
**11CATJ322** ; 178
a-hbh abhaa ahhab hhhhh bbaa- haaba ahhhd hbabh bhaha ahhdb -hb-- hdhd-
a-ahb dhb-b bbahb hddhd -bbdd ddbbh -bd-b bd-da bbbah -a
**14AGCM098** ; 179
a---c a-caa accaa ccccа ccaac caaca ccccа ccccc ccaca acccc ccca- caccc
acacc accac ccacc ccccc -cccc ccccc -cc-c cc-ca ccccа ca
**CDC2D** ; 180

aaaba hbaaa -h-ah -hbha bbaaa haaba -b-ha bbhbh -haha hhhhb -aba- habab
-ha-a hhbah bb-hb hbhh- -bhhh hhbhh aha-b hh-hh hbbaa hh
**11AGAJ188 ; 181**
a---d hbddh adddd hddha hbadd daaha d-hha bbhhh bhdha hhhhb daba- habdb
ahaha hhbad bhah- hb-hh -bhah -hb-- ----- hh-hh hhbbd -h
**HIRZ ; 182**
aaaba hbaah -h-ah -hhh- hba-a haaha hb-ha bbhhb -haha hhhhh -aba- habhb
ahaha habah bhahb hbhhh -bhah aahhh aha-b hh-hh hbbaa hh
**12ACAJ353 ; 183**
a-c-a ccaac aaaca aaa-a ccaa- caaca cccca cccac ccaca ccccc caca- ccccc
acaca aacaa -caac ccccc -ccac aaccc -ha-b c--cc cbaaa -a
**11CACM332 ; 184**
a-cca ccacc ccacc accca accaa caaca cccca cccac ccacc ccccc c-ca- ccccc
accca aacaa ccacc ccccc -ccac aacc- -ca-c ca-cc cccca cc
**11CACM357 ; 185**
a-ccc ccacc ccacc accca accaa ccaca cccca cccac ccacc ccccc c-ca- ccccc
accca aacaa ccacc ccccc -ccac aacc- -ca-c ca-cc cccca cc
**11CACM349 ; 186**
a-ccc ccacc ccacc accca accaa ccaca cccca cccac ccacc ccccc c-ca- ccccc
accca aacaa ccacc ccccc -ccac aacc- -ca-c ca-cc cccca cc
**11AGGJ311 ; 187**
a-hbh hhdhh dbdhd addha abbad hhaha hbbha hbbab bhahh hhhhb bdba- bhbhb
ahhba aahaa hhahh bbbbh -bhdh aahhh -ha-b ha-hd hbbhd hh
**11CACJ330 ; 188**
a-dbd ddddd dbddd ddddd dbbdd ddddd dbbdd dbbdb bdddd ddddb b-bd- bdbdb
dddbd ddddd ddddd bbbbd -bddd dddd- -dd-b dd-dd dbbdd dd
**11CATM147 ; 189**
a-ccc ccacc ccacc accca acca- ccaca cccca cccac ccaac caccc -ac-- cccc-
accca cac-c ccacc ccccc -ccac aaccc -ca-c ca-cc accca -c
**J292 ; 190**
h-dbh hhhhh cb-hh hbbhh acbhh hhhbh bbbha hbbab bbabc hchhb b-bh- bhbhb
hhcch ahbah hhabh bbbbh -bchb aahb- -ha-b ca-hc bbbba hh
**MIXTA-L1a ; 191**
hhhh- -bhhh aa-bh -hab- aab-h bhaa- -a-ah hhhh- hhahb hahah baha- hbbbh
-hh-h hbhhb hb-aa ahhhh -bbha hbahb bbb-a h--hh ----- --
**MIXTA ; 192**
baha- -abaa -h-ha habh- bh-hh hhhb- -h-hh ahbha -aahh hbbaa -hbh- hhahh
-hhhh hhh-- h---b hha-h -bahb aaaba hhh-a ba-aa a-hha ha
**MIXTA-L3 (b,d) ; 193**
dddd- -dbbd -d-db -dbd- ----b ddbb- -d-dd ddddd -dddd ddddd -bdd- ddddd
-dd-b dddbd d--db ddddd -dddd dbdbd bbd-d b--bd ----- --
**MIXTA-L2 ; 194**
h-a-- --ah- ----- --hh- aa-h- -a--- -a-ha -a--h -ha-- --ahh -ah-- ahhah
-hh-h hah-- h--aa cah-h -ahbd hhhaa haa-h a--c- --caa a-
**MAT5 ; 195**
bhhh- -h-hb -a-aa -hh-- ah--a ahha- -h-aa habah -ahhb bbhha -ahb- abhhh
-hb-a chhha a--ha hhbbb -hhhh hhbah bhb-h a--hb ----- --
**MIXTA-L1b ; 196**
hhhh- -b-hh -a-bh -hab- ----h bhaa- -a-ah hhhhh -hahb hahah bah-- -hhbh
-hb-h hbhhb h--aa ahhhh -bbha hbahb hbb-a b--hh ----- --
**MIXTA-L3CD ; 197**
ahhh- -habh -a-hb -hbh- bb--b hhbb- -h-hh hhhhh -hhhh hhhhh abhh- ahahh
-hh-b hhh-- h--hb hhhhh -hhhh hbhbh bbh-h b-b-h ----- --